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Award Number: DAMD17-02-1-0304

TITLE: The Role of IQGAP1 in Neoplastic Growth and Metastasis

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REPORT DATE: June 2005

TYPE OF REPORT: Annual Summary



PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| 01-06-2005 | Annual Summary | 1 Jun 2002 – 31 May 2005 |

**4. TITLE AND SUBTITLE**

The Role of IQGAP1 in Neoplastic Growth and Metastasis

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
DAMD17-02-1-0304

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

Monideepa Roy, Ph.D.
Dr. David Sacks.

**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

Brigham and Women's Hospital
Boston, MA 02115

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**

The purpose of this proposal is to evaluate the role of IQGAP1 in neoplastic transformation and metastasis of breast epithelial cells. The main emphasis is on whether IQGAP1 is involved in invasion and metastasis of transformed breast epithelial cells, as well as the possible involvement of IQGAP1 in regulating β-catenin function. Major findings are: (i)there is a high level of expression of IQGAP1 in breast epithelium; (ii)overexpression of IQGAP1 in mammalian cells enhances cell migration and proliferation; and (iii) β-catenin function and subcellular distribution were modulated by IQGAP1. These data reveal that IQGAP1 has a fundamental role in cell motility and invasion in breast epithelium. This information could have potential therapeutic implications in patients with breast cancer.

**15. SUBJECT TERMS**
Signal Transduction, Cell Biology, Metastasais, Protein-protein interaction

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | 19b. TELEPHONE NUMBER *(include area code)* |
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## INTRODUCTION

Malignancy results from altered modulation of cell adhesion and motility. IQGAP1 is a critical link in the signal transduction pathways leading to enhancement of cell motility and altered cell-cell adhesion. IQGAP1 appears to act as a focal point of cross-talk where diverse regulatory and structural proteins converge. Importantly, IQGAP1 is significantly increased in highly metastatic melanoma and gastric cells. The hypothesis to be evaluated in this proposal is that elevated levels of IQGAP1 promote neoplastic transformation and metastasis. The role of IQGAP1 in cell motility and proliferation has been examined. In addition, IQGAP1 also interacts with β-catenin, a proto-oncogene that participates in cell-cell adhesion and transcriptional co-activation. The effects of IQGAP1 on the stability, subcellular localization and transcriptional co-activation of β-catenin will be assessed. Elucidation of the role played by IQGAP1 in promoting neoplasia and metastasis will enhance our comprehension of the molecular mechanisms responsible for breast cancer.

## BODY

Research accomplishments are described according to the Tasks listed in the approved Statement of Work.

### Task 1. Ascertain whether IQGAP1 is involved in invasion and metastasis of transformed cells

#### i. Examine IQGAP1 protein levels in cell types of varying malignancy

Our studies involving analysis of selected cell lines revealed that there is a very high level of expression of IQGAP1 in breast epithelium (Fig. 1A). On comparison of MCF-7 and MDA-MB-231 breast cancer cells with MCF10A normal breast cells, we made the unexpected observation that MCF-10A had 4.5- and 3-fold more IQGAP1 than MDA-MB-231 and MCF-7 cells respectively (Fig. 1B and C). In contrast, our collaborator, Richard Hynes at the Center for Cancer Research at MIT observed that IQGAP1 mRNA levels in metastatic melanoma cells are higher than those in non-metastatic cells (1). These findings suggest that the level of IQGAP1 expression is altered in cancer cells.

#### ii. Determine if IQGAP1 overexpression promotes tumour cell migration

Migration of MCF-7 cells stably transfected with pcDNA3 vector (termed MCF/V cells) and MCF-7 cells stably transfected with pcDNA3-IQGAP1 (termed MCF/I cells) expressing IQGAP1 at three times the levels expressed in MCF/V cells was evaluated. As shown in our first report (Annual report dated June 2003), MCF/I cells exhibited a 2.8 ± 0.17-fold (mean ± S.E., $n$=16, $p$<0.005) greater motility than MCF/V cells (Fig. 2). Similarly, transient overexpression of IQGAP1 accelerated motility by 1.6 ± 0.07-fold ($n$=4, $p$<0.005) and 1.7 ± 0.09-fold ($n$=4, $p$<0.005) in

HEK-293H cells and highly motile MDA-MB-231 cells, respectively (Fig. 3). The role of IQGAP1 in increased cell motility implies that IQGAP1 could also contribute to cell invasion. Our results revealed that MCF/I cells were 2.5-fold more invasive than MCF/V cells (Fig. 4).

**iii. Determine if IQGAP1 overexpression promotes tumour cell proliferation**

As outlined in our first report (June 2003) equal numbers of MCF/V and MCF/I cells were examined for their [$^3$H]thymidine uptake to measure DNA synthesis and therefore, cell proliferation. Our analysis reveals that, compared to MCF/V cells, MCF/I cells increased [$^3$H]thymidine uptake.

**iv. Develop antisense oligonucleotides to inhibit IQGAP1 expression and analyze the effect of decreased IQGAP1 protein levels on cell migration and proliferation**

Originally, antisense oligonucleotides were proposed to inhibit IQGAP1 protein expression. However, after submission of the original proposal, small interfering RNA (siRNA) was developed. This technique is an efficient method to specifically knock down individual proteins in mammalian cells. Because RNA interference offers many advantages over antisense oligos, we adopted this approach to disrupt IQGAP1 function in cells. Several siRNA oligonucleotides were designed, targeting different regions of IQGAP1. We observed that transient expression of an siRNA complementary to basepair (bp) 4959–4977 of IQGAP1 reduced IQGAP1 protein levels by over 50% (Fig. 5A). Importantly, reduction of IQGAP1 protein by siRNA significantly retarded the ability of MCF-7 cells to migrate through Transwell pores (Fig. 5B). In order to verify the specificity of the siRNA, another oligonucleotide was utilized, termed siRNA 9, which is directed against bp 6705–6723 of IQGAP1. Transfection of siRNA 9 significantly reduced both endogenous IQGAP1 (Fig. 5A) and cell migration through Transwell pores (Fig. 5B). By contrast, siRNAs 2, 3, 5, and 6, which do not reduce IQGAP1 protein expression, had no effect on cell motility (Fig. 5A and B).

We employed a retroviral system to stably integrate siRNA 8 into the genome of MCF-7 cells. IQGAP1 protein expression in these cells (termed MCF-siIQ8 cells) was reduced by 80% (Fig. 6A). Compared with native MCF-7 cells, cell migration of MCF-siIQ8 cells was decreased by 71% (Fig. 6B). The magnitude of reduction of cell migration correlated with the extent of the decrease in IQGAP1 protein levels. These data strongly suggest that IQGAP1 is required for cell motility.

Since cell motility is influenced by growth factors (2) we looked at mitogen activated protein (MAP) kinase signalling. We showed that both overexpression and knockdown of IQGAP1 reduced the ability of EGF to stimulate ERK phosphorylation (Fig. 7).

Because IQGAP1 functions as a scaffolding protein in several signalling pathways (3, 4), we hypothesized that IQGAP1 might serve as a scaffold in the MAP kinase signalling cascade. If correct, one would anticipate that IQGAP1 would modulate activation of MEK, the molecule immediately upstream of ERK in the ERK/MAP kinase cascade. This concept was evaluated initially by examining EGF–stimulated phosphorylation of MEK in cells expressing different levels of IQGAP1. To stably downregulate IQGAP1 in MCF/V, MCF/I and MCF-siIQ8 cells.The relative amounts of (active) phospho-MEK were determined by probing blots with an antibody that specifically recognizes phosphorylated MEK. EGF stimulated MEK phosphorylation by 3.47- ± 0.6-fold (mean ± S.E., $n$=3) in MCF/V cells (Fig. 8). Modulation of intracellular IQGAP1 levels impaired the ability of EGF to induce MEK phosphorylation. Following EGF treatment, levels of phospho-MEK in MCF/I cells were significantly lower ($p<0.001$) than those in MCF/V cells (Fig. 8). The ability of EGF to induce MEK phosphorylation was also markedly attenuated by knockdown of IQGAP1 in MCF-siIQ8 cells.

The effect of IQGAP1 on EGF-stimulated MEK activity was confirmed in MCF-7 cells transiently expressing siRNA 8. MEK phosphorylation in these cells was 50% lower than in vector-transfected cells. By contrast, siRNA 5 - which is directed against a different region of IQGAP1 and does not reduce IQGAP1 protein expression (Fig. 9A) - had no effect on MEK phosphorylation (Fig. 9). Analogous to the results with MCF/I cells, transient overexpression of IQGAP1 in MCF-7 cells significantly reduced activation of MEK by EGF (Fig. 9). These results are analogous to our prior observations with ERK where both overexpression and knockdown of IQGAP1 significantly reduced EGF stimulation of ERK activity. Our findings suggested that an optimal level of intracellular IQGAP1 is required for maximal activation of EGF-stimulated MAP kinase signalling.

**Task 2. Test the hypothesis that IQGAP1 regulates β-catenin function in breast cancer cell lines of varying malignancy**

*Effect of IQGAP1 Expression on β-Catenin Stability* -- Several proteins that alter the function of β-catenin act by changing its rate of degradation (5). Therefore, the effect of IQGAP1 on the half-life of β-catenin was examined in MCF-7 cells transfected with either vector or IQGAP1. The degradation of β-catenin was monitored by [$^{35}$S]methionine pulse-chase labeling and the $t_{1/2}$ of β-catenin was estimated from the slope of the decay curve. The $t_{1/2}$ of β-catenin in MCF-7 cells was ~2.5 h, and transient transfection of IQGAP1 did not significantly alter the degradation rate of β-catenin (data not shown).

*IQGAP1 Stimulates β-Catenin Function in Transcriptional Co-activation*-- The effect of IQGAP1 on the transcriptional co-activator function of β-catenin was examined with luciferase assays (6). pTop-flash and pFop-flash, the firefly luciferase reporter plasmids used in this study, contain wild type and mutant TCF/LEF binding sites, respectively. Therefore, β-catenin-mediated co-activation of TCF/LEF transcription was measured from the ratio of pTop-flash to pFop-flash luciferase units (6). These

reporter plasmids were transiently transfected into MCF-7 cells with both an *R. reniformis* luciferase plasmid (to control for transfection efficiency) and vector (pcDNA3) or wild type IQGAP1. Overexpression of IQGAP1 in MCF-7 (Fig. 10A) cells had no significant effect on β-catenin-mediated TCF/LEF signaling. It is possible that the effects of IQGAP1 on β-catenin transcriptional co-activation can be detected only in cells containing stabilized β-catenin. To examine this hypothesis, SW480 colon cancer cells were used that harbor a mutation in the *APC* gene rendering the APC protein defective in β-catenin turnover (7). The transcriptional co-activator function of β-catenin was examined in SW480 cells transiently transfected with reporter plasmids and either vector or IQGAP1, as described for MCF-7 cells. Compared with vector, transient overexpression of IQGAP1 in SW480 cells enhanced β-catenin-mediated TCF/LEF signaling by $1.7 \pm 0.08$-fold (mean ± S.E., $n = 12, p < 0.0001$). By contrast, overexpression of IQGAP1ΔC (that does not bind β-catenin) had no significant effect on β-catenin-mediated TCF/LEF signaling (Fig. 10B), suggesting that binding of IQGAP1 to β-catenin was necessary for the effect on β-catenin function.

*Overexpression of IQGAP1 Alters the Subcellular Distribution of β-Catenin--* Transient overexpression of IQGAP1 enhanced β-catenin-mediated transcriptional co-activation and had little effect on the turnover of soluble β-catenin, suggesting that IQGAP1 might enhance translocation of β-catenin into the nucleus. This hypothesis was examined by immunocytochemistry and laser confocal microscopy. As overexpression of IQGAP1 altered β–catenin function in SW480 cells, these cells were transfected with a dual-promoter plasmid expressing both green fluorescent protein (GFP) and myc-IQGAP1, probed for β-catenin and stained with DAPI to highlight nuclei. Cells transfected with GFP fluoresced *green* (Fig. 11, *left panel*). Probing cells with anti-myc antibody confirmed co-expression of myc-IQGAP1 in GFP-transfected cells, and IQGAP1 was not observed to accumulate in the nucleus (data not shown). β-Catenin in SW480 cells, highlighted in the *second panel* of Fig. 11 ("*Total β-catenin*"), is distributed predominantly in the cytoplasm and nucleus. SW480 cells transfected with IQGAP1 demonstrated a modest, but statistically significant ($34 \pm 9.5\%$, mean ± S.E., $n = 40, p < 0.05$) increase in the amount of β-catenin in the nucleus compared with non-transfected cells (Fig. 11).

## KEY RESEARCH ACCOMPLISHMENTS

- there is a high level of expression of IQGAP1 in breast epithelium
- overexpression of IQGAP1 in mammalian cells enhances cell migration
- IQGAP1 overexpressing MCF-7 cells are more invasive than vector-transfected MCF-7 cells
- overexpression of IQGAP1 enhances cellular proliferation
- decreased IQGAP1 protein levels reduced cell motility
- IQGAP1 regulates mitogen-activated protein (MAP) kinase signalling
- IQGAP1 stimulates β-catenin-mediated transcriptional co-activation
- Overexpression of IQGAP1 alters the subcellular distribution of β-catenin

## REPORTABLE OUTCOMES

**Manuscripts:**

1. Roy, M., Li, Z., Sacks, D. B. IQGAP1 binds ERK2 and modulates its activity. Journal of Biological Chemistry, *279:* 17329-37, 2004.

2. Roy, M., Li Z., Sacks D. B. IQGAP1 is a scaffold for MAP kinase signalling. Mol Cell Biol: in press, 2005.

**Abstracts:**

1. Roy, M., Li, Z., Sacks, D. B. IQGAP1 Interacts with Erk2 and Modulates Mitogen-Activated Protein (MAP) Kinase Signalling. 12th International Conference on Second Messengers and Phosphoproteins, August 2004, Montreal
(Category: Signaling Networks)

2. Roy, M., Li, Z., Sacks, D. B. IQGAP1 binds ERK2 and modulates its activity. ASBMB Annual Meeting and 8th IUBMB Conference, June 2004, Boston
(Category: Integration of Signaling Mechanisms Meeting)

3. Roy, M., Li, Z., Sacks, D. B. IQGAP1 is a Scaffold for Mitogen-Activated Protein (MAP) Kinase Signalling. Breast Cancer Research at Harvard, 2$^{nd}$ Symposium, March 25, 2005, Boston

## CONCLUSIONS

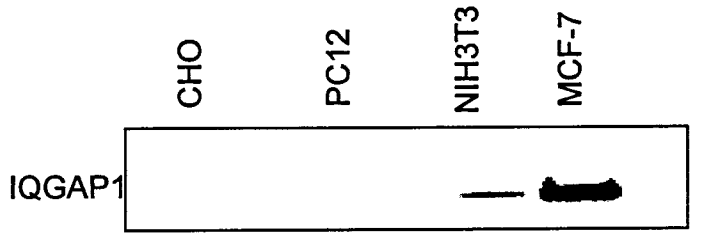
The work performed to date has yielded some insights into the role of IQGAP1 in cell motility. We observed that there is a high level of IQGAP1 protein expressed in breast epithelium. In addition, overexpression of IQGAP1 in transformed breast cells enhanced cell migration and cell proliferation. In contrast, decreased IQGAP1 levels reduced cell motility. β-catenin function and subcellular distribution were modulated by IQGAP1. Initial findings support the hypothesis that IQGAP1 plays a significant role in the metastasis of breast epithelial cells. This information could help determine whether IQGAP1 is a potential therapeutic target for breast cancer.

## REFERENCES


1. Clark EA, Golub TR, Lander ES, Hynes RO. Genomic analysis of metastasis reveals an essential role for RhoC. Nature 2000; 406:532-5.
2. Briggs MW, Sacks DB. IQGAP proteins are integral components of cytoskeletal regulation. EMBO reports 2003; 4:571-4.
3. Briggs MW, Sacks DB. IQGAP1 as signal integrator: $Ca^{2+}$, calmodulin, Cdc42 and the cytoskeleton. FEBS Lett 2003; 542:7-11.
4. Wells A. Tumor invasion: role of growth factor-induced cell motility. Adv Cancer Res 2000; 78: 31-101.

5. Polakis, P. The oncogenic activation of beta-catenin. Curr. Opin. Genet. Dev. 1999; 9:15-21
6. van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M., and Clevers, H. Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell 1997; 88:789-799
7. Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and Polakis, P. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc. Natl. Acad. Sci. U. S. A. 1995; 92:3046-3050

A

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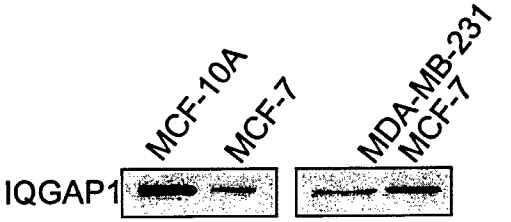


C

| Cell line | Amount (ng/μg) of lysate |
|---|---|
| MCF-10A | 0.225 |
| MDA-MB-231 | 0.05 |
| MCF-7 | 0.075 |

**Fig. 1.** *A,* Comparison of the amount of IQGAP1 among cells. Equal amounts of protein lysate from the indicated cell lines were analyzed for IQGAP1 by Western blotting. *B,* Comparison of the amount of IQGAP1 among breast cells. Equal amounts of protein lysate were processed as described in *A*. *C,* Western Blots were scanned and densitometry was performed using UN-SCAN-IT software. Calibration of bands was done by running known amounts of protein on the same gel.

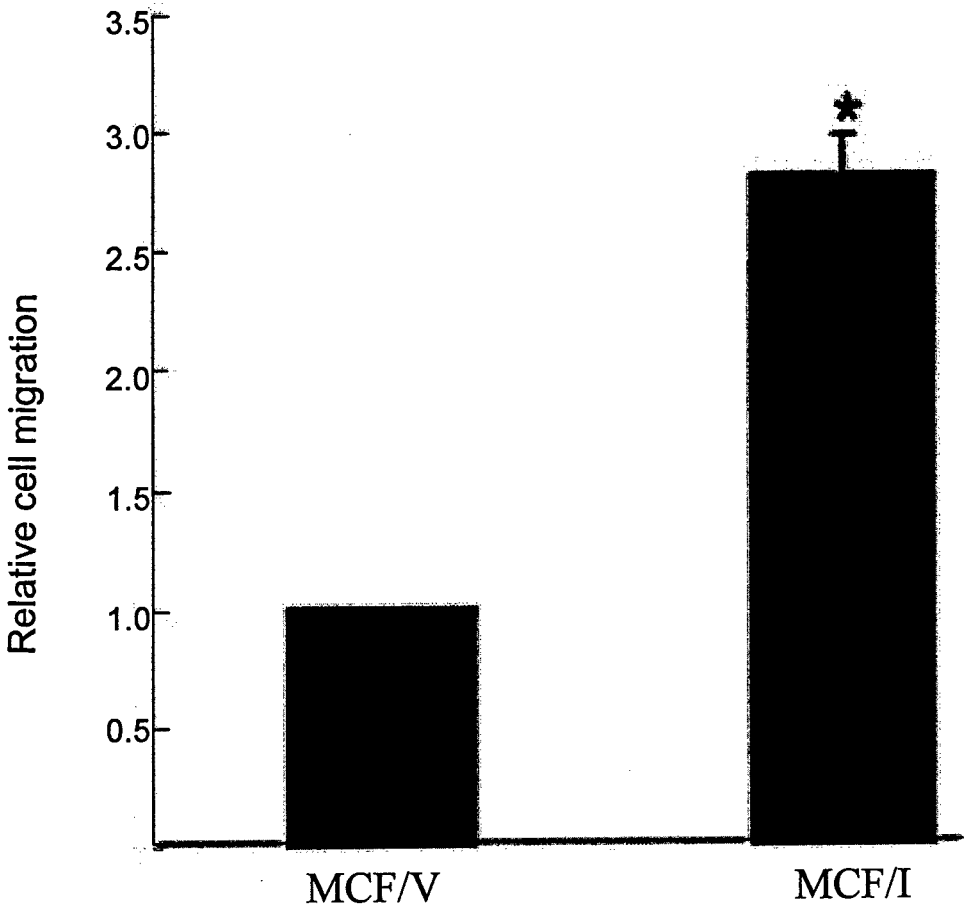


**Fig. 2.** Migration of MCF/V and MCF/I cells through Transwell pores was quantified by counting fields of migratory cells under a light microscope. Data, expressed relative to migration of MCF/V cells, represent the means ± S.E. ($n$ = 16). *, significantly different from MCF/V ($p < 0.005$).

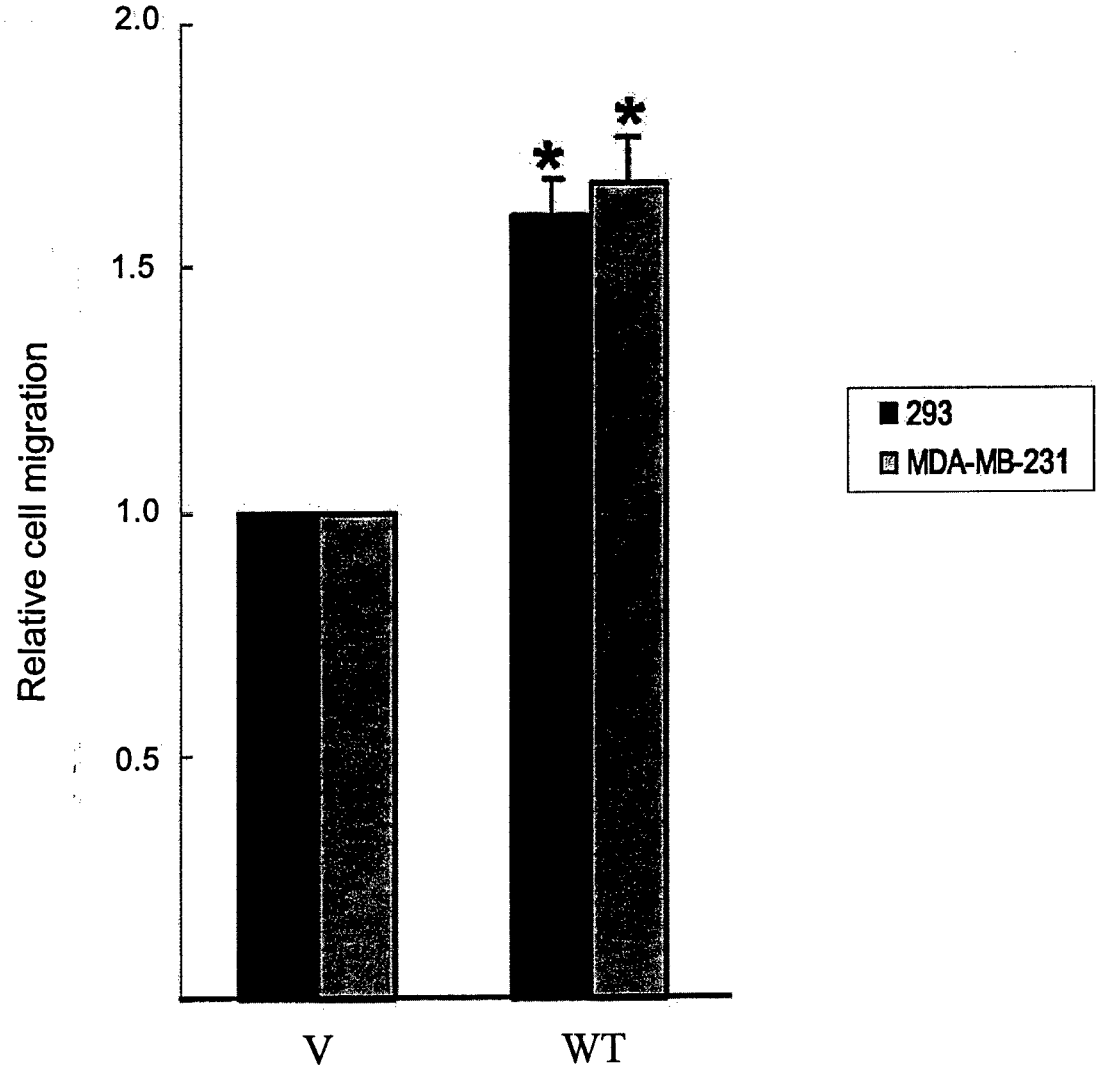


**Fig.3.** Migration through Transwells of HEK-293H and MDA-MB-231 cells transiently transfected with vector (*V*) or IQGAP1 (*WT*) was examined. Data are expressed relative to the migration of vector-transfected cells and represent the means ± S.E. ($n$=4). *, significantly different from vector-transfected cells ($p < 0.005$).

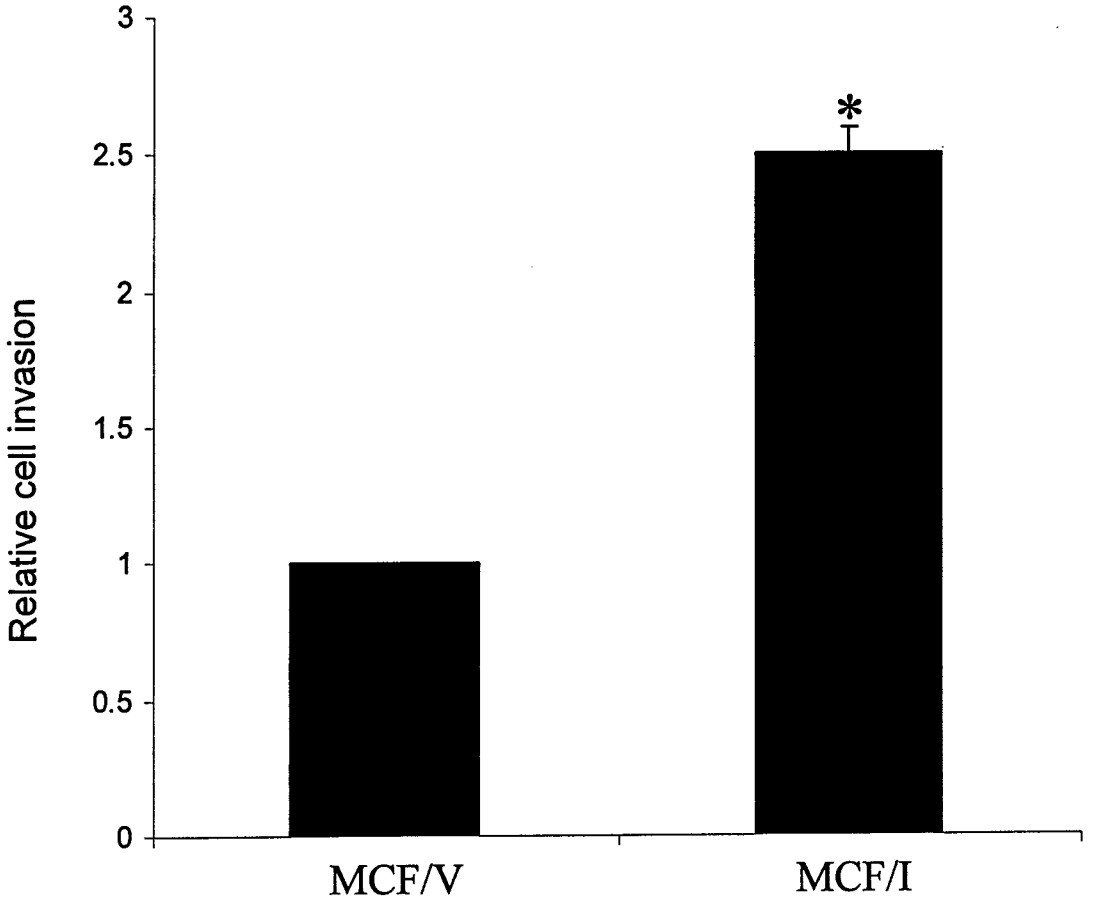


**Fig. 4.** Invasion of MCF/V and MCF/I cells was quantified by counting fields of migratory cells under a light microscope. Data, expressed relative to migration of MCF/V cells, represent the means ± S.E. ($n$ = 4). *, significantly different from MCF/V ($p < 0.005$).

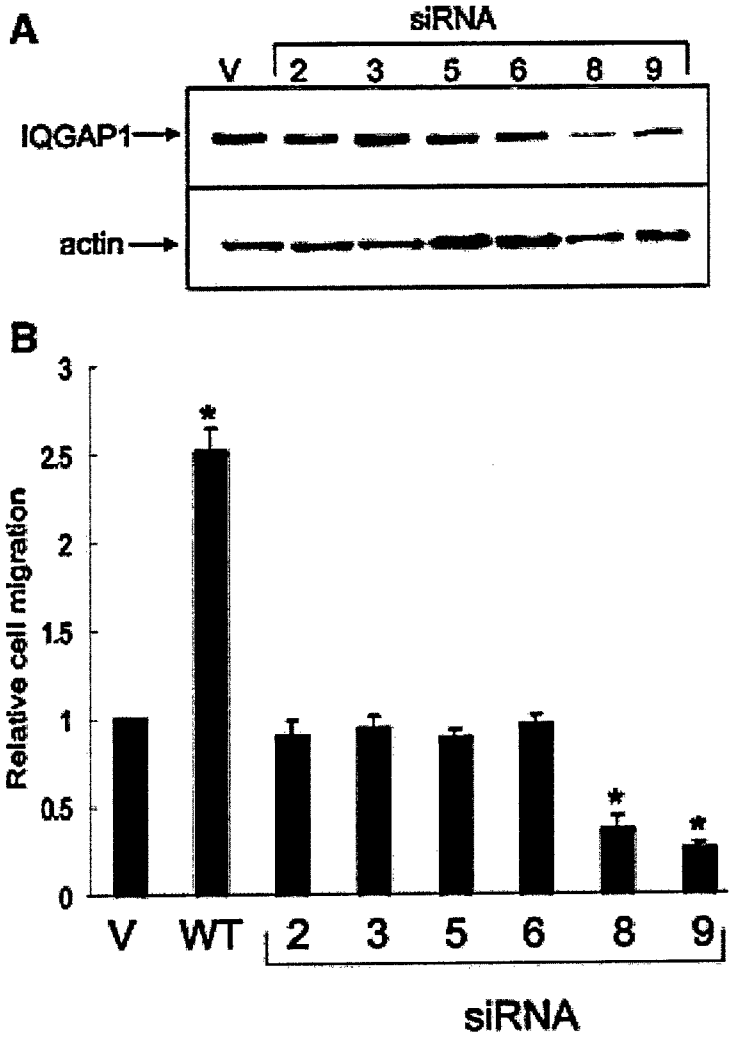


**Fig. 5. Reduction of IQGAP1 by siRNA attenuates cell migration.** *A*, MCF-7 cells were transiently transfected with mU6pro (*V*) or mU6siRNA 2, 3, 5, 6, 8, or 9. Equal amounts of protein lysates were analyzed by Western blotting with anti-IQGAP1 and anti-actin antibodies. *B*, MCF-7 cells were transfected with vector (*V*), IQGAP1 (*WT*), or mU6siRNA 2, 3, 5, 6, 8, or 9. Migration data, expressed relative to vector-transfected cells, represent the means ± S.E. ($n$ = 6 for vector, wild type, and mU6siRNA 8, and $n$ = 2 for mU6siRNA 2, 3, 5, 6, and 9). *, significantly different from vector-transfected cells ($p < 0.001$).

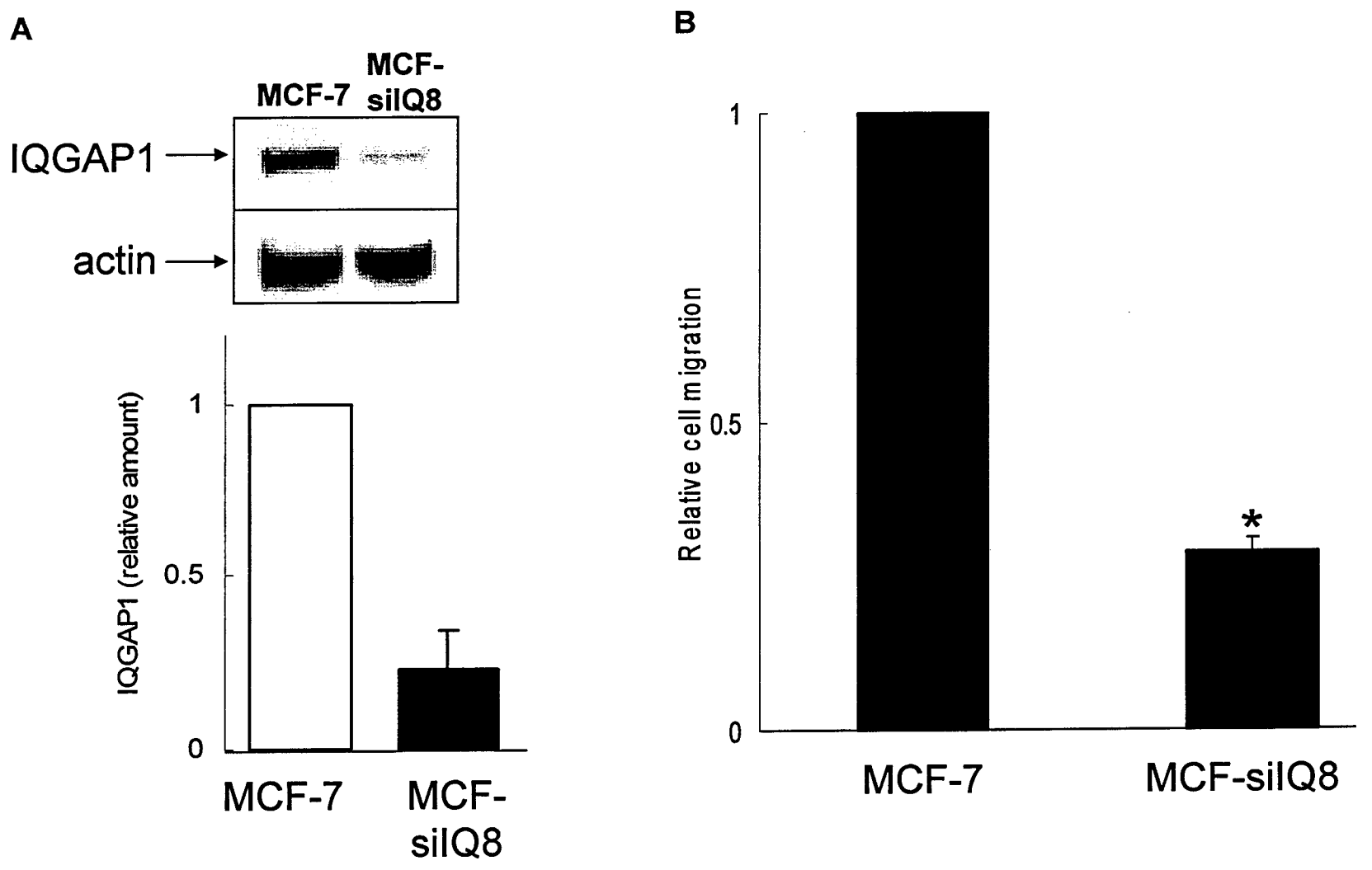


**Fig. 6. Stable expression of siRNA for IQGAP1 reduces cell motility.** *A*, equal amounts of protein lysates from MCF-7 and MCF-siIQ8 cells were analyzed by Western blotting with anti-IQGAP1 and anti-actin antibodies. IQGAP1 protein, quantified by densitometry and corrected for the amount of actin, is expressed relative to MCF-7 cells ($n = 2$). *B*, migration of MCF-7 and MCF-siIQ8 cells. Migration data, expressed relative to MCF-7 cells, represent the means ± S.E., $n = 3$. *, significantly different from MCF-7 cells ($p < 0.0001$).

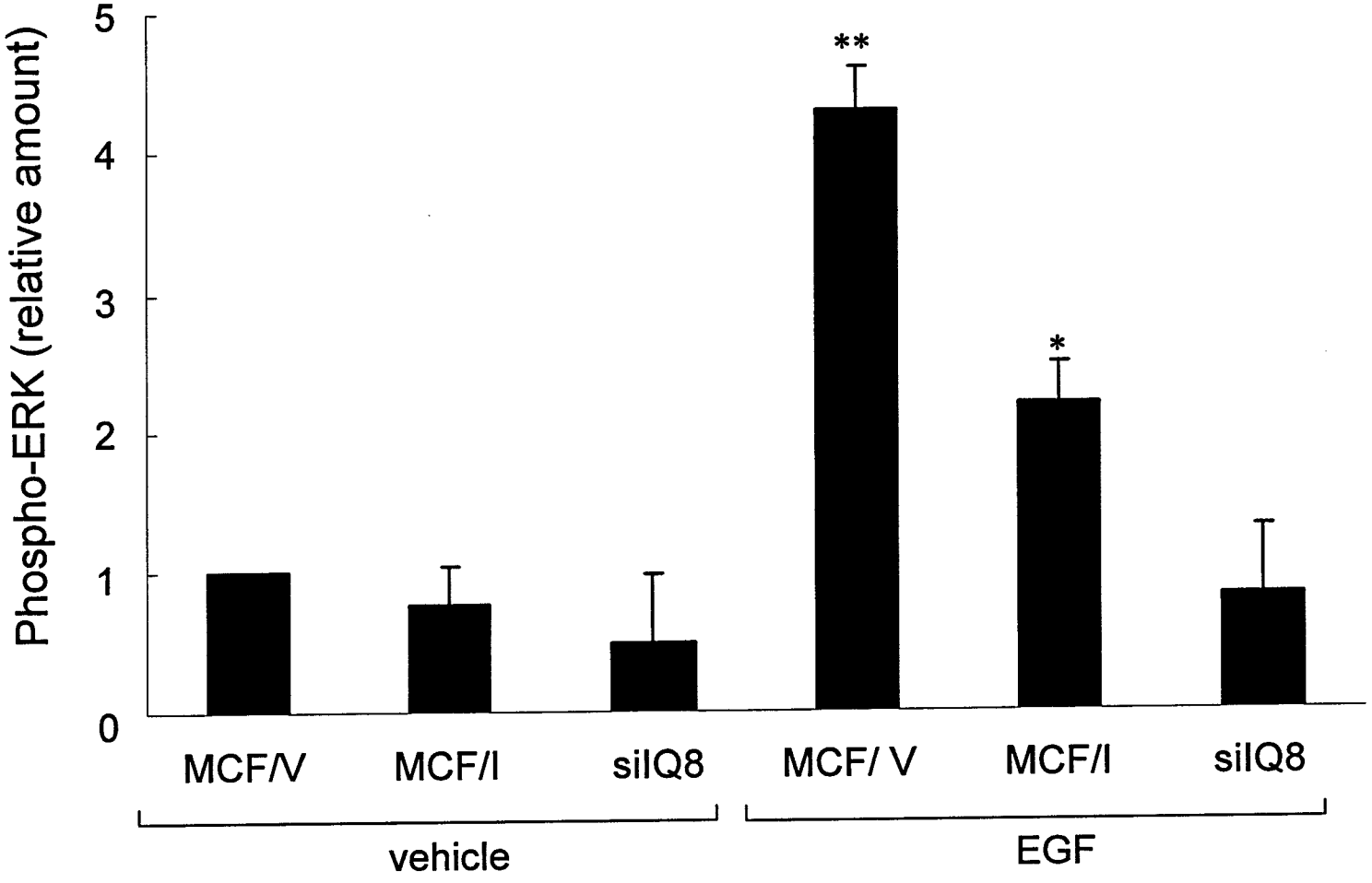


**Fig. 7. IQGAP1 modulates EGF-stimulated ERK activity.** ERK2 activity was examined in MCF-7 cells stably expressing either vector (MCF/V), IQGAP1 (MCF/I) or siRNA for IQGAP1 (siIQ8). Cells were starved of serum overnight and treated with vehicle or 100 ng/ml EGF for 10 min. The amount of phosphorylated ERK isoforms (Phospho-ERK) was quantified by densitometry and corrected for the amount of ERK2 in the corresponding lysate. Data, expressed relative to the amount of phospho-ERK in vehicle-treated MCF/V cells, represent the means ± S.E. ($n$=4). *, significantly different from vehicle-treated MCF/V cells ($p<0.01$). **, significantly different from vehicle-treated MCF/V cells ($p<0.001$).

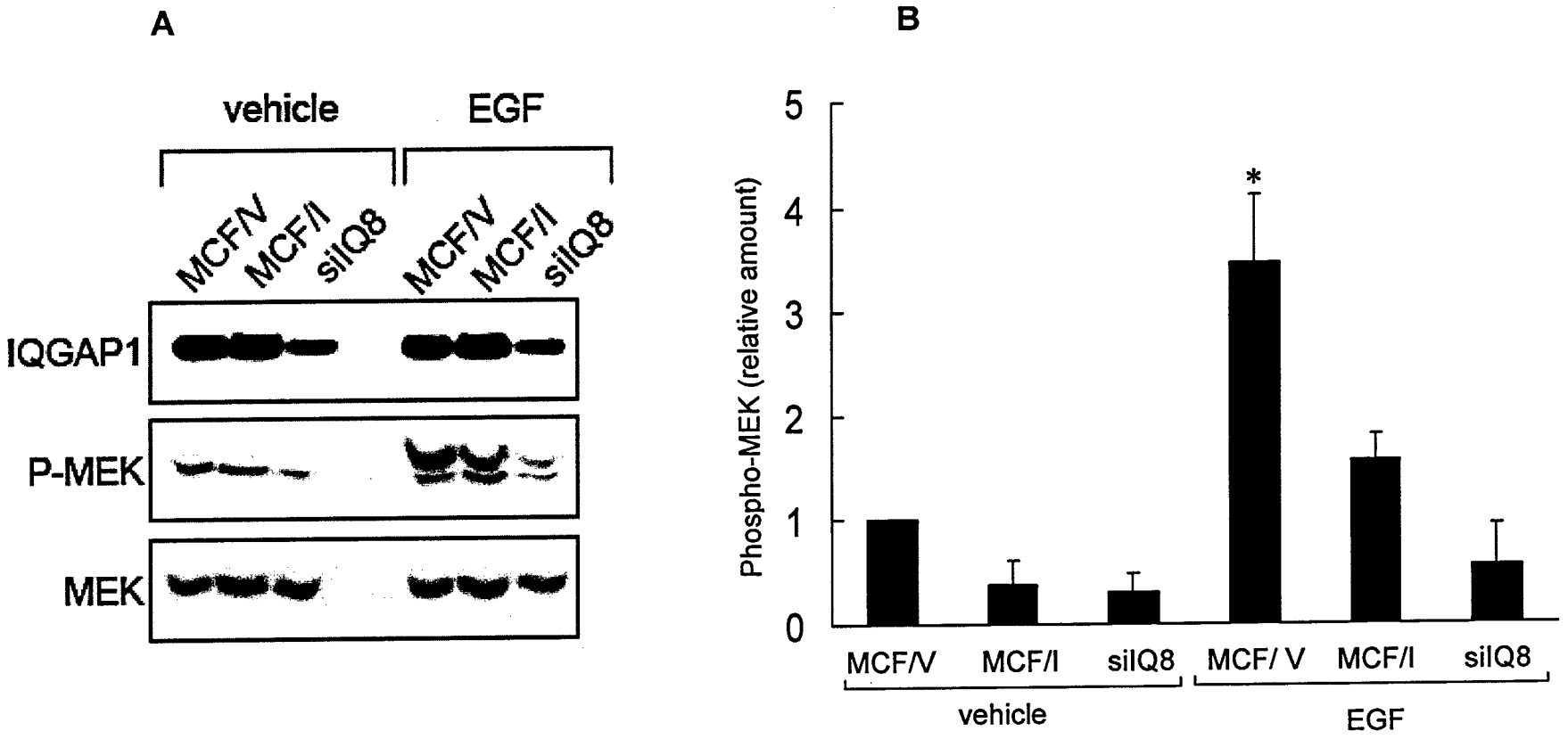


**Fig. 8. IQGAP1 modulates EGF-stimulated activation of MEK.** *A,* MCF/V, MCF/I and MCF-siIQ8 (siIQ8) cells were starved of serum overnight and treated with vehicle or 100 ng/ml EGF for 10 min. Equal amounts of protein from the cell lysates were resolved by SDS-PAGE and Western blots were probed with anti-IQGAP1 and anti-phospho-MEK antibodies. The membranes were stripped and reprobed with anti-MEK antibody. *B,* the amount of phospho-MEK was quantified by densitometry and corrected for the amount of MEK in the corresponding lysate. Data, expressed relative to the amount of phospho-MEK in vehicle-treated MCF/V cells, represent the means ± S.E. ($n$=3). *, significantly different from vehicle-treated MCF/V cells ($p<0.001$).

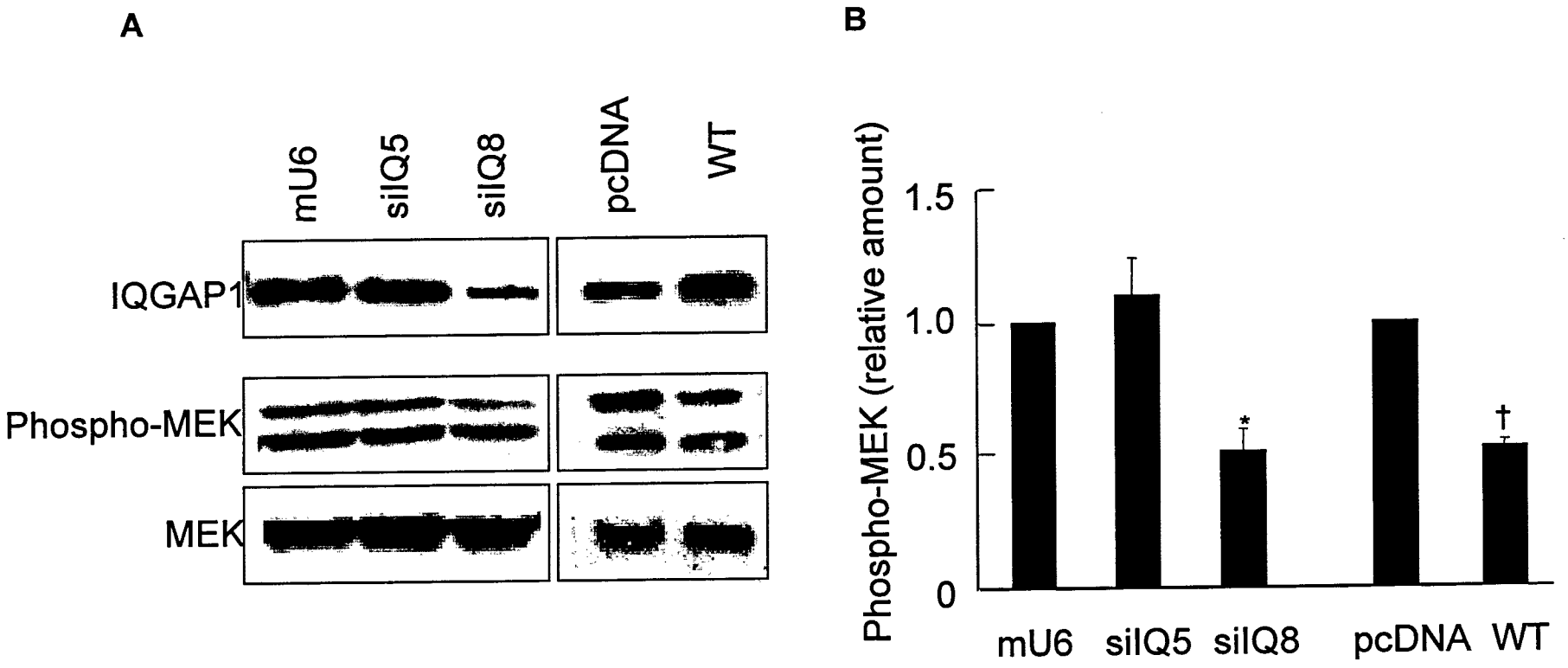


**Fig. 9. Altering intracellular IQGAP1 concentrations reduces EGF-stimulated MEK activity.** *A,* MCF-7 cells were transiently transfected with vectors mU6pro (mU6) or pcDNA3 (pcDNA), IQGAP1 (WT) or siRNAs for IQGAP1 (siIQ5 and siIQ8). Cells were processed as described for Fig. 1A. *B,* the amount of phospho-MEK was quantified by densitometry and corrected for the amount of MEK in the corresponding lysate. Data, expressed relative to the amount of phospho-MEK in cells transfected with the appropriate vector, represent the means ± S.E. ($n$=3). *, significantly different from mU6-transfected cells ($p<0.001$); †, significantly different from pcDNA-transfected cells ($p<0.01$).

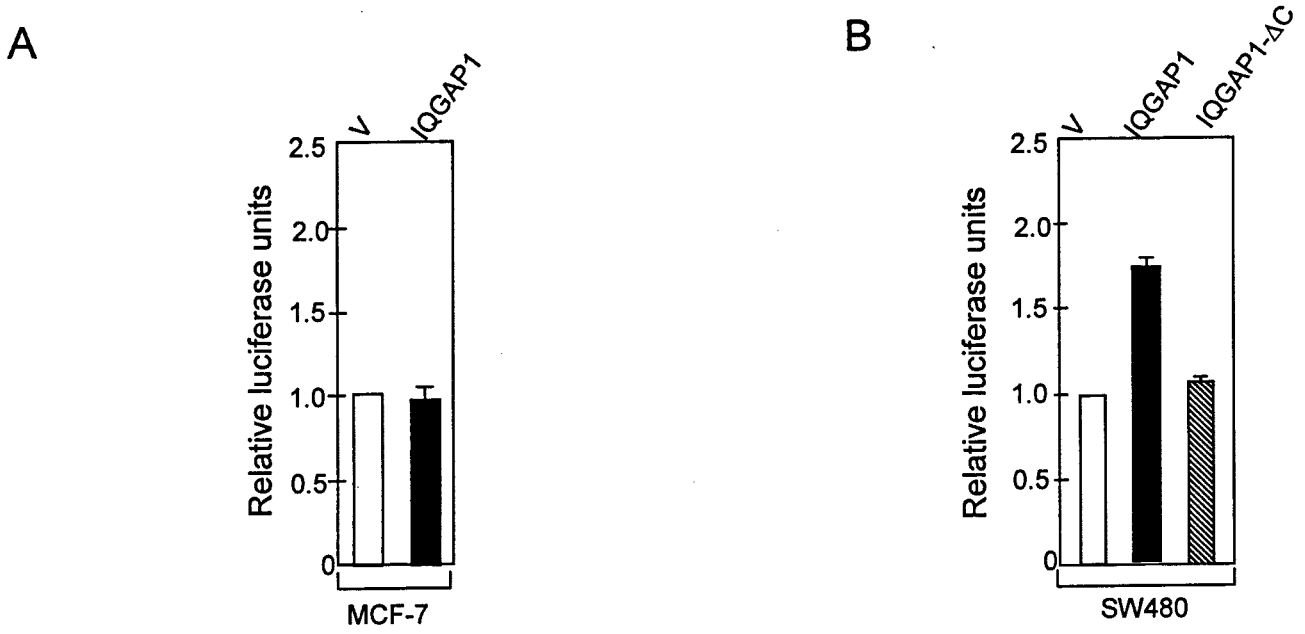


**Fig. 10. IQGAP1 stimulates stabilized β-catenin.** *A,* MCF-7 cells stably transfected with either vector (*V, open bar*) or IQGAP1 (*solid bar*) were transiently transfected with either pTop-flash or pFop-flash. pRL-TK was co-transfected to normalize for transfection efficiency. Twenty-four hours after transfection, cells were lysed, and luciferase activities were determined by luminometry. Data, expressed as relative luciferase units (pTop-flash units divided by mutant pFop-flash units), are the mean of twelve (wild type IQGAP1) or three (IQGAP1- C) separate experiments, each performed in triplicate. Means ± S.E. are shown. Results are expressed relative to cells transfected with vector alone, which was set as 1.0. *Asterisk*, significantly different from vector alone ($p < 0.0001$). *B,* SW480 cells were transiently transfected with either vector (*V, open bar*), IQGAP1 (*solid bar*), or IQGAP1-ΔC ( *C, hatched bar*) and co-transfected with reporter plasmids and analyzed as described in panel A.

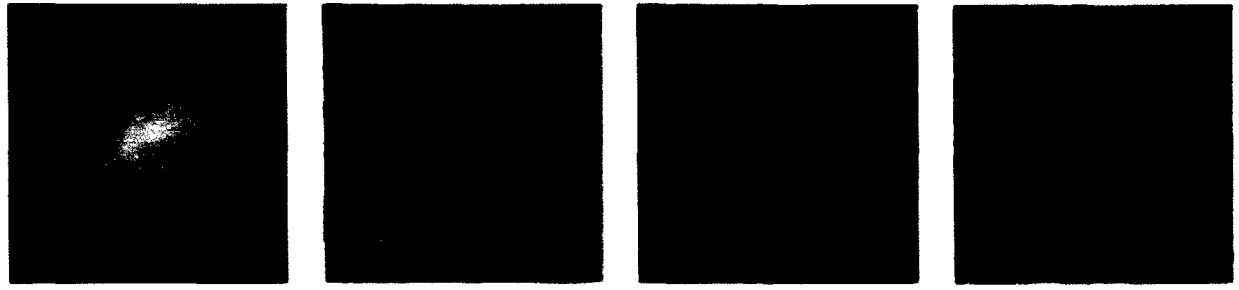


**Fig. 11. IQGAP1 overexpression alters the subcellular distribution of β-catenin.** SW480 cells seeded onto slides were transfected with a dual-promoter plasmid expressing both GFP and myc-IQGAP1 and processed for immunocytochemistry. Cells transfected with the dual-promoter construct expressing IQGAP1 and GFP fluoresced *green* (*GFP*). Cells were probed with anti-β-catenin antibody and visualized with TRITC-labeled secondary antibody, which fluoresced *red* (*Total β-catenin*). In addition, the cells were co-stained with DAPI to highlight the nuclei (*DAPI*), and the *blue* and *red channels* were merged (*Nuclear β -catenin*).

# IQGAP1 is a scaffold for MAP kinase signalling[‡]

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Running Title: IQGAP1 modulates MAPK signaling

## Abstract

IQGAP1 modulates many cellular functions such as cell-cell adhesion, transcription, cytoskeletal architecture and selected signalling pathways. We previously documented that IQGAP1 binds extracellular signal-regulated kinase (ERK) 2 and regulates growth factor-stimulated ERK activity. Here we show that MEK, the molecule immediately upstream of ERK in the Ras/MAP kinase signalling cascade, also interacts directly with IQGAP1. Both MEK1 and MEK2 bound IQGAP1 *in vitro* and co-immunoprecipitated with IQGAP1. Addition of ERK2 enhanced by fourfold the *in vitro* interaction of MEK2 with IQGAP1 without altering binding of MEK1. Similarly, ERK1 promoted MEK binding to IQGAP1, but neither MEK protein altered the association between IQGAP1 and ERK. Epidermal growth factor (EGF) differentially regulated binding, enhancing MEK1 interaction while reducing MEK2 binding to IQGAP1. In addition, both knockdown and overexpression of IQGAP1 reduced EGF-stimulated activation of MEK and ERK. Analyses with selective IQGAP1 mutant constructs indicated that MEK binding is crucial for IQGAP1 to modulate EGF activation of ERK. Our data strongly suggest that IQGAP1 functions as a molecular scaffold in the Ras/MAP kinase pathway.

## Introduction

The mitogen activated protein (MAP) kinase cascades, which are activated in response to a variety of extracellular stimuli, mediate signal transduction from the cell surface to the nucleus (9, 36). The most widely studied MAP kinase pathway is the extracellular signal-regulated kinase (ERK) cascade. ERK has an important role in the transduction of several proliferative and differentiative signals in normal cellular development and oncogenesis. The components of the ERK module form a linear cascade consisting of Raf (MAP3K), MEK (MAP or ERK kinase-MAP2K) and ERK (MAPK) kinases.

Diverse MAP kinase pathways respond to different external cues and exert distinct biological functions. However, there seems to be potential for extensive overlap, not only with regard to the activating stimuli, but also with regard to downstream targets. The mechanism by which specificity and fidelity of distinct biological responses are maintained is largely unknown. Recent reports indicate that specificity in MAP kinase signalling in yeast is attained predominantly by scaffolding proteins (38). Similarly, a number of scaffolding proteins that modulate signalling from Raf to MEK and ERK have been identified in mammalian cells (for reviews, see (23, 36)). These include kinase suppressor of Ras (KSR) (37), connector enhancer of KSR (CNK) (19), Sur8 (28), MEK partner1 (MP1) (42), MAP kinase organizer 1 (MORG1) (48), Sef (45) and Raf kinase inhibitor protein (RKIP) (51).

IQGAPs are multidomain molecules that contain several protein-interacting motifs (for reviews, see (4, 5)). The name is derived from the presence of an IQ domain (with four tandem IQ motifs) and a region with significant sequence similarity to the

catalytic domain of Ras-GAPs. Other motifs include a calponin homology domain (CHD), a coiled-coil region and a WW domain. The multiple conserved modules in IQGAP1 suggest that it participates in protein-protein interactions and may be a component of several signaling pathways. This hypothesis has been validated by both our group and others who documented that IQGAP1 binds activated Cdc42 and Rac1 (but not RhoA or Ras) (11, 16, 20), actin (10, 17, 32), calmodulin (17, 20, 32), E-cadherin (25, 29), β-catenin (3, 25), S100B (33), nectin (21), CLIP-170 (12), ERK2 (40) and adenomatous polyposis coli (49). The diversity of IQGAP1 targets has led to the hypothesis that IQGAP1 functions as a scaffolding protein that can assemble multiprotein complexes within various subcellular domains (4, 17). In support of this concept, published evidence reveals that IQGAP1 is capable of binding several proteins simultaneously. For example, complexes of IQGAP1 with actin and Cdc42 have been isolated *in vitro* (10). Moreover, complexes of IQGAP1 with Cdc42 and calmodulin (Ho, 99), Rac1 and calmodulin (41), or Rac1/Cdc42 and CLIP-170 (12) have been reported. These findings strongly suggest that IQGAP1 functions as a scaffold within cells.

We previously demonstrated that IQGAP1 binds directly to ERK2 and regulates its activity (40). Moreover, altering intracellular IQGAP1 levels inhibited epidermal growth factor (EGF)-stimulated activation of ERK. These findings led to the question of whether IQGAP1 may modulate MEK, the molecule immediately upstream of ERK in the Ras-Raf-MEK-ERK pathway. Here, we present evidence that IQGAP1 and MEK associate both *in vitro* and in intact cells. Analogous to our observations with ERK, the ability of EGF to stimulate phosphorylation of MEK was abrogated in cells in which endogenous IQGAP1 was specifically knocked down by small interfering RNA (siRNA). Both overexpression and specific knockdown of endogenous IQGAP1 significantly

impaired EGF-stimulated MEK and ERK activities. These data suggest that IQGAP1 participates as a scaffolding protein in MEK-ERK signalling.

## Materials and Methods

*Materials*- Tissue culture reagents were obtained from Life Technologies, Inc. Fetal bovine serum was purchased from Biowhittaker. Anti-phospho-ERK antibody was obtained from Promega. Anti-ERK, anti-MEK and anti-phospho-MEK antibodies were obtained from Cell Signalling Technology. Anti-HA antibody was purchased from Santa Cruz Biotechnology. Anti-myc monoclonal antibody was manufactured by Maine Biotechnology. The anti-IQGAP1 polyclonal antibody has been previously characterized (17). The anti-IQGAP1 monoclonal antibody was a generous gift from Andre Bernards (MGH Center for Cancer Research, Charlestown, MA). Secondary antibodies for enhanced chemiluminescence (ECL) detection were from Amersham Pharmacia Biotech. All other reagents were of standard analytical grade.

*Plasmid Construction and Expression*- Myc-tagged human IQGAP1 in pcDNA3 vector (17) and plasmids pMCL-MKK1 and pMCL-MKK2, for expression of HA-tagged MEK1 and MEK2, respectively were used for expression in mammalian cells. For *in vitro* studies, the NpT7-5 $His_6$-ERK1 (39) NpT7-5 $His_6$-ERK2 (39), pRSET $His_6$-MEK1 (30) and pRSET $His_6$-MEK2 (30) constructs were expressed in *E. coli* and purified by nickel affinity chromatography using $Ni^{2+}$-NTA agarose (Qiagen). GST fusion proteins of IQGAP1 were also expressed in *Escherichia coli* and isolated with glutathione-Sepharose as previously described (17). All proteins were >90% pure by Coomassie Blue staining. All plasmids were purified using Qiagen DNA Purification Kits (Qiagen), according to the manufacturer's instructions.

*Cell lines*- MCF-7 human breast epithelial cells which stably overexpress either pcDNA3 (termed MCF/V) or pcDNA3-myc-IQGAP1 (termed MCF/I cells) have been described previously (3, 44). MCF/I cells have 3-fold more IQGAP1 than MCF/V cells (44). Stable expression of siRNA for IQGAP1 was described earlier (31). The IQGAP1 protein level in these cells (termed MCF-siIQ8 cells) is reduced by 80%.

*Cell Culture and Transfection*- MCF-7 cells and HEK-293H (Gibco-BRL) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. MCF-7 cells were transfected using FuGENE 6 (Roche Molecular Biochemicals) as previously described (29). HEK-293H cells were transfected using Lipofectamine 2000 (Gibco-BRL) according to the manufacturer's instructions. Nucleofection of MCF-7 cells with HA-tagged MEK1 and MEK2 was performed using Nucleofector solution V (Amaxa) and the program P-20 according to the manufacturer's instructions.

*Transient Knockdown of IQGAP1* -Transient knockdown of IQGAP1 by siRNA was carried out as previously described (31). Briefly, siRNA directed against IQGAP1, cloned into the mU6pro vector (mU6siRNA 5 or 8), and mU6pro vector alone were transfected into MCF-7 cells with FuGENE6. After 24 h, cells were processed as described below for measurement of MAP kinase activity.

*Measurement of MAP kinase activity*- Equal numbers of MCF-7 cells were incubated in DMEM containing 0.5% serum at 37 °C. Sixteen hours later, 100 ng/ml EGF or an equal volume of vehicle was added for 10 min at 25 °C. The reactions were terminated by

removing the media, washing the cells twice with phosphate-buffered saline (PBS) at 4 °C, and lysing in buffer A (20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol and 0.2% Triton X-100) containing phosphatase inhibitor cocktail II (Sigma). ERK activity was assayed by Western blotting cell lysates using an antibody specific for phosphorylated ERK to detect phospho (active) ERK (40). MEK is activated by phosphorylation on two serine residues. In order to monitor MEK activity, Western blots of lysates were probed with anti-phospho-MEK antibody that detects active (phosphorylated) MEK. In all cases, cell lysates were equalized for protein concentration prior to SDS-PAGE. Where indicated, blots were stripped by incubating in 62.5 mM Tris, pH 6.8, 2% (w/v) SDS, and 0.7% (v/v) β-mercaptoethanol for 30 min at 50 °C and re-probed with anti-ERK or anti-MEK antibodies to measure total ERK amd MEK, respectively. Blots were also probed with anti-myc and anti-IQGAP1 antibodies as specified.

*Crosslinking of proteins-* MCF-7 cells, starved of serum, were incubated with EGF or vehicle for 10 min. Where indicated, 1 mM dithiobis succinimidyl propionate (DSP) (Pierce) in PBS was added, followed 10 min later with Tris (pH 7.4) to a final concentration of 50 mM. DSP reacts with primary amine groups and crosslinks bound proteins. The cells were washed with PBS 15 min later and lysed in buffer A. Immunoprecipitation was performed as described below.

*Immunoprecipitation-* MCF-7 cells were transiently transfected with HA-MEK1 or HA-MEK2. After 24 h, cells were starved of serum, followed by incubation with EGF where indicated (see below). Immunoprecipitation was performed essentially as previously described (29). Equal amounts of protein lysate were incubated for 3 h at 4 °C with anti-

IQGAP1 polyclonal antibody immobilized on protein A-Sepharose (Amersham) beads. Samples were washed five times with buffer A, and Western blotting was performed. Immunoprecipitation with non-immune rabbit serum (NIRS) was performed in parallel. Blots were probed with anti-IQGAP1 monoclonal antibody and anti-MEK antibodies, and antigen-antibody complexes were identified with horseradish peroxidase-conjugated sheep-anti-mouse and donkey-anti-rabbit antibodies, respectively. ECL was used for detection.

*TNT product production and binding analysis-* [$^{35}$S]methionine-labeled TNT (transcription and translation) products were produced with the TNT Quick Coupled Transcription/Translation system (Promega) according to the manufacturer's instructions. The construction of IQGAP1 and IQGAP1-C (C-terminal region, amino acids 864-1657), IQGAP1-N1 (comprising amino acids 2-431), IQGAP1-N2 (amino acids 432-864), IQGAP1 (IQGAP1ΔWW, amino acids 643-744 deleted), IQGAP1ΔIQ, amino acids 699-905 deleted, and IQGAP1ΔCHD, amino acids 35-365 deleted, were described previously (17, 40, 43). Briefly, 0.5 μg of the IQGAP1 plasmids were incubated with 40 μl TNT Quick Master Mix (Promega) and 20 μCi of [$^{35}$S]methionine (New England Nuclear) at 30 °C for 1 h. Products were confirmed by SDS-PAGE and autoradiography.

For *in vitro* binding experiments with [$^{35}$S]methionine-labeled products, the IQGAP1 constructs described in the previous paragraph were incubated with $His_6$-MEK1 or $His_6$-MEK2 in 500 μl of buffer A for 3 h at 4 °C. Samples were washed six times in buffer A, complexes were isolated with $Ni^{2+}$-NTA agarose and resolved by SDS-PAGE. Bands were analyzed after autoradiography of the dried gel.

*In vitro binding*- GST (1 μg) or GST-IQGAP1 (~0.7 μg) bound to glutathione-Sepharose was incubated with equimolar quantities of $His_6$-MEK1 or $His_6$-MEK2 in 500 μl of buffer A for 3 h at 4 °C. Where indicated, equivalent amounts of $His_6$-ERK1 or $His_6$-ERK2 was added. After centrifugation, samples were washed, resolved by SDS-PAGE and the gel was cut in half; the top portion (containing IQGAP1) was stained with Coomassie Blue and the bottom half was transferred to polyvinylidene fluoride (PVDF) membrane and blots were probed with anti-MEK antibody. Blots were stripped thereafter by incubating in 62.5 mM Tris, pH 6.8, 2% (w/v) SDS, and 0.7% (v/v) β-mercaptoethanol for 30 min at 50 °C and reprobed with anti-ERK antibody.

*Miscellaneous*- Densitometry of ECL signals was analyzed with UN-SCAN-IT software (Silk Scientific Corporation). Statistical analysis was assessed by Student's *t* test, with InStat software (GraphPad Software, Inc.). Protein concentrations were determined with the DC protein assay (Bio-Rad).

## Results

*IQGAP1 binds MEK1 and MEK2 in vitro-* Analysis with pure proteins was used to examine a possible interaction between IQGAP1 and MEK1 or MEK2. A GST fusion protein of full length IQGAP1 expressed in *E. coli* was incubated with bacterially produced, recombinant, purified $His_6$-MEK1 or $His_6$-MEK2 and complexes were isolated with glutathione-Sepharose. Analysis by Western blotting revealed that MEK1 and MEK2 each bound to IQGAP1 (Fig. 1). Binding was specific as neither MEK protein was detected in the samples incubated with GST alone. Coomassie staining of the top half of the gel revealed that approximately equivalent amounts of GST-IQGAP1 were present (Fig. 1, upper panel).

*IQGAP1 binds MEK in cells-* To ascertain whether endogenous IQGAP1 interacts with MEK in a normal cell milieu, immunoprecipitation was performed. MCF-7 cells were transiently transfected with HA-MEK1 or HA-MEK2 using Nucleofector (Amaxa) as described under "Experimental procedures". Immunoprecipitating lysates with anti-IQGAP1 antibody revealed that endogenous IQGAP1 bound both MEK1 and MEK2 (Fig. 2A). In unstimulated cells, the amount of MEK2 that co-immunoprecipitated with IQGAP1 was slightly more than the amount of MEK1. No MEK1 or MEK2 was detected in samples immunoprecipitated with non-immune rabbit serum, confirming that IQGAP1 and MEK specifically interact *in vivo*.

EGF activates the MAPK cascade, enhancing both MEK and ERK activity (18, 27). We previously observed that incubation of cells with EGF did not change the amount of ERK that co-immunoprecipitated with IQGAP1 (40). To determine whether activating

MEK modulates its interaction with IQGAP1, MCF-7 cells were stimulated with EGF for 10 min prior to lysis and immunoprecipitation. EGF enhanced the binding of MEK1 to IQGAP1 by 6-fold (Fig. 2A). In contrast, under identical assay conditions, EGF reduced by 3.5-fold the amount of MEK2 that co-immunoprecipitated with IQGAP1. Our results reveal that IQGAP1 and MEK associate in human breast epithelial cells and that EGF differentially modulates the interaction of MEK1 and MEK2 with IQGAP1.

*ERK1 co-immunoprecipitated with IQGAP1.* Immunoprecipitation was performed to determine whether endogenous ERK1 binds endogenous IQGAP1 in cells. Immunoprecipitation with anti-IQGAP1 antibody revealed that ERK1 bound to endogenous IQGAP1 (Fig. 2B). Binding was specific as no ERK1 was detected in samples immunoprecipitated with non-immune rabbit serum. Incubation with EGF did not significantly change the amount of ERK1 that bound to IQGAP1 (Fig. 2B). These results indicate that, analogous to ERK2 (40), ERK1 associates with IQGAP1 in human breast epithelial cells and this interaction is not altered by EGF.

*IQGAP1 modulates EGF-stimulated activation of MEK-* We have previously documented that IQGAP1 binds ERK and regulates its activity (40). Because IQGAP1 functions as a scaffolding protein in several signalling pathways (4, 5, 17), we hypothesized that IQGAP1 might serve as a scaffold in the MAP kinase signalling cascade. If correct, one would anticipate that IQGAP1 would modulate activation of MEK, the molecule immediately upstream of ERK in the ERK/MAP kinase cascade. This concept was evaluated initially by examining EGF–stimulated phosphorylation of MEK in cells expressing different levels of IQGAP1. MCF-7 cells that stably

overexpress IQGAP1 (termed MCF/I cells) or empty vector (MCF/V cells) (3, 44) were used. MCF/I cells express 3-fold more IQGAP1 than MCF/V cells (Fig. 3A and Ref. (3)). To stably downregulate IQGAP1 in MCF-7 cells, we used a retroviral vector to integrate a specific siRNA for human IQGAP1 into the genome. The IQGAP1 protein level in these cells (termed MCF-siIQ8 cells) was reduced by 80% (Fig. 3A and Ref. (31)). The relative amounts of (active) phospho-MEK were determined by probing blots with an antibody that specifically recognizes MEK phosphorylated at Ser217/219. EGF stimulated MEK phosphorylation by 3.47- ± 0.6-fold (mean ± S.E., $n$=3) in MCF/V cells (Fig. 3). Modulation of intracellular IQGAP1 levels impaired the ability of EGF to induce MEK phosphorylation. Following EGF treatment, levels of phospho-MEK in MCF/I cells were significantly lower ($p<0.001$) than those in MCF/V cells (Fig. 3). The ability of EGF to induce MEK phosphorylation was also markedly attenuated by knockdown of IQGAP1 in MCF-siIQ8 cells. These results are analogous to our prior observations with ERK where both overexpression and knockdown of IQGAP1 significantly reduced EGF stimulation of ERK activity (40).

The effect of IQGAP1 on EGF-stimulated MEK activity was confirmed in transiently transfected cells. Transient transfection of siRNA 8 in MCF-7 cells reduced IQGAP1 protein expression by 70-80% (Fig. 4A). MEK phosphorylation in these cells was 50% lower than in vector-transfected cells. By contrast, siRNA 5 - which is directed against a different region of IQGAP1 and does not reduce IQGAP1 protein expression (Fig. 4A) - had no effect on MEK phosphorylation (Fig. 4). Analogous to the results with MCF/I cells, transient overexpression of IQGAP1 in MCF-7 cells significantly reduced activation of MEK by EGF (Fig. 4).

*Identification of the MEK1 and MEK2 binding domain on IQGAP1-* The MEK binding domain on IQGAP1 was investigated using deletion mutants and fragments of IQGAP1. Selected constructs of IQGAP1 (Fig. 5) were labelled with [$^{35}$S]methionine in a reticulocyte lysate and incubated with $His_6$-MEK1 or $His_6$-MEK2. His-tagged MEK proteins were isolated by $Ni^{2+}$ affinity chromatography and the fragments of IQGAP1 that bound were resolved by SDS-PAGE and identified by autoradiography. [$^{35}$S]methionine-labelled full length IQGAP1 bound to $His_6$-MEK1 and to $His_6$-MEK2 (Fig. 6A and B). The lower molecular weight bands are degradation fragments of IQGAP1. Specificity of binding was verified by the absence of IQGAP1 from samples with $Ni^{2+}$ beads alone (Fig. 6A and B). Examination of the two halves of IQGAP1 revealed that only the N-terminal half (amino acid residues 1-863) bound to MEK1 and MEK2; no interaction between the C-terminal half of IQGAP1 and MEK was observed (Fig. 6A and B). Inspection of the input (Fig. 6C) indicates that approximately equal amounts of the N- and C-terminal halves were incubated with MEK. Similarly, the amounts of $His_6$-MEK1 and $His_6$-MEK2 in each sample were essentially identical (data not shown).

In order to narrow the binding site, the N-terminal portion of IQGAP1 was divided into two equal halves, termed N1 and N2 (Fig. 5). Both $His_6$-MEK1 and $His_6$-MEK2 bound N2 exclusively (Fig. 6A and B), revealing that the binding domain is between residues 432 and 863 of IQGAP1. In addition, the identified protein interaction domains in the N-terminal half of IQGAP1 were deleted (Fig. 5). Analysis of binding showed that neither removal of the CHD (amino acids 37-265 deleted) nor the WW region (amino acids 643-744 deleted) from IQGAP1 attenuated its binding to MEK1 or MEK2 (Fig. 6A and B). In contrast, absence of the region that includes the IQ domain

(amino acids 699-905 deleted) completely disrupted the interaction of IQGAP1 with MEK. These data demonstrate that the IQ region of IQGAP1 is necessary for MEK binding. The input for each IQGAP1 construct is depicted in Fig. 6C.

*Effects of IQGAP1ΔIQ and IQGAP1ΔWW on MEK and ERK activities-* We previously documented that transient overexpression of full length IQGAP1 in MCF-7 and HEK 293H cells significantly reduced activation of ERK1 and ERK2 by EGF (40). Therefore, we determined the effect of wild type and mutant IQGAP1 constructs on MEK activity. Transient transfection of HEK-293H cells with wild type IQGAP1 reduced EGF-stimulated MEK activity by 55% (Fig. 7A and B). Similarly, transfection of IQGAP1ΔWW, which binds MEK with an affinity similar to that of wild type IQGAP1 (see Fig. 6A and B), significantly reduced EGF stimulation of MEK. In contrast, transfection of an equivalent amount of IQGAP1ΔIQ, which lacks the MEK binding domain, did not impair the ability of EGF to activate MEK (Fig. 7A and B). These data reveal that interaction of MEK with IQGAP1 is necessary for IQGAP1 to modulate EGF-stimulated MEK activity.

In an earlier report, we showed that IQGAP1ΔWW - which does not bind ERK - had no effect on EGF-stimulated ERK activation (40). [Note that when IQGAP1ΔWW is expressed at very high levels (exceeding approximately 10 times endogenous IQGAP1), ERK activity is progressively reduced (data not shown). This effect is probably due to sequestration of MEK by IQGAP1ΔWW, preventing the normal coupling of MEK to ERK that is mediated by endogenous IQGAP1.] To extend these observations, we examined whether ERK activity was affected by deleting the MEK binding domain from

IQGAP1. Although IQGAP1ΔIQ binds normally to ERK (40), transfection of IQGAP1ΔIQ to the same level as wild type IQGAP1 did not change the level of phosphorylated ERK upon EGF stimulation (Fig. 8A and B). These data indicate that binding to IQGAP1 regulates the ability of MEK to activate ERK.

*Effect of ERK on the binding of MEK to IQGAP1 -In vitro* analysis with pure proteins was conducted to examine whether ERK1 or ERK2 has any effect on the binding of IQGAP1 to MEK1 or MEK2 and vice versa. A GST fusion protein of full length IQGAP1 was incubated with purified $His_6$-MEK1 or $His_6$-ERK1 separately, or with $His_6$-MEK1 and $His_6$-ERK1 together. Complexes were isolated with glutathione-Sepharose and the relative amount of binding was compared by Western blotting. ERK1 increased the binding of MEK1 to IQGAP1 by threefold (Fig. 9A). In contrast, the presence of MEK1 did not significantly alter the interaction of ERK1 with IQGAP1. Staining the upper portion of the gel with Coomassie Blue indicated that equal amounts of GST-IQGAP1 were present (Fig. 9). Specificity of the interactions was revealed by the absence of bands from samples incubated with GST alone. Both ERK1 (Fig. 9B) and ERK2 (Fig. 10A) enhanced the interaction of MEK2 with IQGAP1 by fourfold. However, ERK2 had no effect on the binding of MEK1 to IQGAP1 (Fig. 10B). Note that neither MEK1 nor MEK2 modulated the interaction of ERK1 or ERK2 with IQGAP1 (Figs. 9 and 10). These data reveal that ERK1 promoted the binding of MEK1 and MEK2 to IQGAP1, while ERK2 specifically enhanced the interaction of MEK2 with IQGAP1.

*IQGAP1 modulates EGF-stimulated activation of ERK and MEK in a concentration-dependent manner-* Because IQGAP1 participates, at least in part, in MEK and ERK signalling, we assessed whether IQGAP1 modulated MEK and ERK activities in a dose-dependent manner. To obtain different expression levels, IQGAP1 was transiently overexpressed or knocked down (by siRNA). Serum-starved cells were stimulated with EGF and lysates were analysed by Western blotting. We observed that EGF was able to activate ERK only within a very narrow range of IQGAP1 concentrations close to endogenous levels (Fig. 11). Interestingly, increasing and decreasing intracellular IQGAP1 levels reduced activation of ERK by virtually identical amounts. Both overexpression and knockdown of endogenous IQGAP1 by ≥ 30% impaired by >65% the ability of EGF to stimulate ERK activity (Fig. 11). Similarly, maximal stimulation of MEK phosphorylation by EGF occurred only when intracellular IQGAP1 concentration was close to endogenous levels (Fig. 11). Increasing or reducing IQGAP1 markedly attenuated the ability of EGF to activate MEK. These data lead us to speculate that, analogous to other scaffolding proteins of the MAP kinase cascade (36, 45, 48), a functional and productive signalling unit can be assembled only when the relative stoichiometries of the kinases and IQGAP1 are optimal.

## Discussion

IQGAP1 binds a diverse array of proteins including calmodulin, Cdc42, Rac1, actin, β-catenin, E-cadherin and CLIP-170 (4). Interaction with IQGAP1 regulates the function of its target proteins (reviewed in (4, 5)). For example, IQGAP1 inhibits cell-cell adhesion mediated by the E-cadherin-β-catenin complex (25, 29), increases β-catenin-mediated transcriptional activation (3) and captures growing microtubules via CLIP-170 at the leading edge of migrating fibroblasts (12). Prior work from our laboratory documented that IQGAP1 binds ERK2 and modulates its activity (40). In this study, we investigated the interaction with IQGAP1 of MEK1 and MEK2, the kinases immediately upstream of ERK, and explored the functional sequelae in the ERK/MAP kinase cascade.

We observed direct binding between IQGAP1 and MEK1, as well as between IQGAP1 and MEK2. Association in cells was revealed by co-immunoprecipitation of MEK1 and MEK2 with endogenous IQGAP1 from epithelial cell lysates. Analogous to the effects on its other targets (4), IQGAP1 modulated MEK function. Changing intracellular concentrations of wild type IQGAP1 impaired the ability of EGF to activate MEK, but a mutant IQGAP1 construct that is unable to bind MEK had no effect. These findings confirm that the binding of IQGAP1 to MEK regulates its activation by EGF.

MAP kinases are evolutionarily conserved enzymes that connect cell-surface receptors to critical regulatory targets within cells (8). Mammals express at least four distinctly regulated groups of MAP kinases. Specificity in MAP kinase activation is secured by several mechanisms. The kinase components of a particular MAP kinase module may interact stepwise through a series of sequential binary interactions creating a

signaling cascade. These kinases may be structurally organized into signalling complexes for proper triggering of the cascade. Alternatively, a scaffold protein may intervene and tether the different components of the cascade together to create a functional module. Scaffolding proteins were originally identified in yeast (14), but a large body of evidence reveals that scaffolds also contribute to the regulation of MAP kinase pathways in mammalian cells (8). For example, KSR, MP1, β-arrestins and MEK kinase 1 are important scaffolding molecules that are involved in regulating ERK activity (36). KSR1 and MP1 contribute to the normal regulation of ERK activity, implying that scaffolding proteins are used in a non-redundant manner. In addition, different scaffolds may permit the MAP kinase signaling complexes to target specific substrates (36). Thus, selected scaffolding proteins mediate diverse MEK/ERK functions. Based on the experimental evidence suggesting that IQGAP1 functions as a scaffolding protein that integrates diverse signaling pathways (4, 5), we hypothesized that IQGAP1 may be an unrecognized scaffold for the Ras/MAP kinase signalling pathway. The evidence presented here supports this hypothesis.

IQGAP1 binds directly to at least two components of the MAP kinase pathway, namely MEK and its target ERK. These interactions modulate the ability of EGF to activate both MEK and ERK. Although necessary, by themselves these factors are not sufficient to establish that IQGAP1 functions as a scaffold in MEK/ERK signaling. Validation of the scaffold concept is provided by the data obtained with selected mutant IQGAP1 constructs. Analysis was performed with IQGAP1ΔIQ, which binds ERK but not MEK, and IQGAP1ΔWW, which binds MEK but not ERK. Transfection of IQGAP1ΔIQ had no effect on activation of MEK or ERK by EGF. In contrast, overexpression of IQGAP1ΔWW attenuated EGF-stimulated MEK activity to the same

extent as that produced by wild type IQGAP1. However, IQGAP1ΔWW did not alter the ability of EGF to activate ERK (Fig. 8B and (40)). Thus, binding of both MEK and ERK to IQGAP1 is required for IQGAP1 to modulate EGF-stimulated ERK activity. These data support the postulate that MEK phosphorylates and thereby activates ERK in a processive manner where both the molecules are bound simultaneously to the adaptor molecule (26).

Increasing and decreasing intracellular IQGAP1 concentrations produced the same outcome, namely marked impairment of stimulation of MEK and ERK by EGF. Maximal activation of MEK and ERK by EGF was observed only when cellular IQGAP1 concentrations were close to normal levels. Deviation by ≥ 50% from these values reduced active MEK and ERK by >50%. Although these findings initially seem counter-intuitive, they actually support the notion that IQGAP1 is a scaffold that links MEK to ERK. This concept is illustrated schematically in Fig. 12. IQGAP1 acting as a scaffold can assemble its client proteins (MEK and ERK) only when all components are present in an appropriate stoichiometric ratio. The concentrations of IQGAP1, MEK2 and ERK2 in MCF-7 cells were determined to be 795 nM, 6.92 μM and 15.45 μM, respectively. These values correspond to relative stoichiometries of IQGAP1:MEK2:ERK2 of 1:9:20. Thus, relatively small changes in the amount of IQGAP1 will result in large changes in the relative stoichoimetries of IQGAP1 to MEK and ERK. When IQGAP1 is in excess, nonfunctional binary complexes of IQGAP1 with only one of the components of the kinase cascade are formed. This model is consistent with published data which reveal that overexpression of the scaffold KSR inhibited ERK-dependent biological effects (6) and high concentrations of MP1 decreased MEK1-ERK1 binding (42). Our data derived from overexpression of IQGAP1 are analogous to these findings. Computer modeling of

the MAP kinase cascade further supports these observations and suggests that increasing or reducing the scaffold concentration decreases maximal activation of the MAP kinase cascade (26). In support of the latter concept, activation of ERKs by EGF was reduced by 50% in KSR-deficient mouse fibroblasts (37). These results are consistent with our data on knockdown of IQGAP1. *In vivo* scaffold levels can be regulated by modulation of gene expression or protein stability, or via sequestration in specific subcellular compartments (22). Although we manipulated intracellular IQGAP1 concentrations in this study, IQGAP1 is not uniformly distributed in the cell. Increased levels of IQGAP1 are found in discrete subcellular locations (29, 34). Moreover, several stimuli, including manipulation of intracellular $Ca^{2+}$ concentrations (32, 33), alteration of calmodulin function (29) and activation of E-cadherin (29), induce the movement of IQGAP1 between different subcellular regions. It is plausible therefore that changes in IQGAP1 concentrations in microdomains of the cell can influence MAP kinase in distinct signaling compartments.

The biological functions of ERK1 and ERK2 are usually considered to be equivalent. Similarly, potential functional differences between MEK1 and MEK2 are rarely explored. A possible explanation for the lack of discrimination in the past between the role of MEK1 versus MEK2 and ERK1 versus ERK2 is the inability of pharmacologic inhibitors to distinguish between these kinases. This limitation is exemplified by the recent demonstration using specific siRNAs that MEK2 and ERK2, but not MEK1 and ERK1, mediate programmed cell death (7). Moreover, older evidence also suggests that MEK1 and MEK2 may not have fully overlapping functions. For example, only MEK1 is activated by serum in some cell lines (50). MEK1 knock-out leads to embryonic lethality from defects in placental vascularization (13), while mouse

growth and development are independent of MEK2 (1). A very recent report disclosed that the two MEK subtypes have different effects on cell cycle progression (46). Analogous observations have been made for ERK. In rat fibroblasts v-Raf selectively induced ERK2 activity (24) and ERK1 and ERK2 are regulated by distinct machinery during differentiation of HL-60 cells by 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA) (35).

Notwithstanding this evidence, very little is known about the molecular mechanism underlying the selectivity. It seems reasonable to postulate that, analogous to their ability to separate MEK/ERK signaling from the p38 MAP kinase cascade (36), distinct scaffolding proteins might mediate signaling specificity. This notion has been described for MP1 which binds specifically to MEK1 and ERK1 and facilitates their activation (42). On the basis of data presented here, we propose that IQGAP1 may serve such a function. In quiescent MCF-7 cells, IQGAP1 bound slightly more MEK2 than MEK1 and differences were markedly amplified on EGF stimulation. The binding of MEK1 to IQGAP1 increased, while that of MEK2 was reduced dramatically by EGF. These data reveal that EGF differentially regulates the binding of MEK1 and MEK2 to IQGAP1. By this mechanism, EGF could promote signaling from MEK1 to ERK, while simultaneously attenuating MEK2/ERK signaling. In this context, it is noteworthy that EGF did not alter the binding of either ERK1 (Fig. 2B) or ERK2 (40) to IQGAP1.

The hypothesis that IQGAP1 functions as a scaffold that enables differential regulation of MEK1 and MEK2 is further bolstered by our *in vitro* binding studies. Analysis with purified proteins revealed that ERK2 specifically enhanced binding of MEK2 to IQGAP1, without altering the IQGAP1-MEK1 interaction. In contrast, ERK1 increased the interaction of MEK1 and MEK2 with IQGAP1. Because neither MEK

protein modulated ERK binding to IQGAP1, the increased binding to IQGAP1 is probably not due to ERK-MEK interactions. The most likely mechanism is that ERK1 and ERK2 change the tertiary conformation of IQGAP1, selectively increasing the accessibility of MEK to its binding site. An alteration in the conformation of IQGAP1 on binding calmodulin has been postulated to explain how calmodulin reduces binding of Cdc42 and E-cadherin to IQGAP1 (17, 29). Our *in vitro* observation raises the possibility of a positive feedback loop in cells where association of ERK1 with IQGAP1 enhances its proximity to, and therefore interaction with, MEK1, leading to increased phosphorylation and activation of ERK1. EGF also increases MEK1 binding to IQGAP1, further augmenting MEK1/ERK1 signalling. For MEK2, the pathway would be different. EGF reduced the binding of MEK2 to IQGAP1, while both ERK1 and ERK2 increased the IQGAP1-MEK2 interaction.

The results of these studies raise the intriguing question as to the mechanism by which IQGAP1 mediates the coupling between the EGF receptor and its downstream signalling partners MEK and ERK. One possible model is that IQGAP1 binds directly to components of the MAP kinase cascade upstream of MEK, such as Raf or Ras or even the EGF receptor itself. In this context, the observation that IQGAP1 is recruited to a complex of activated Grb2-EGF receptors (2) is particularly relevant. Prior reports failed to identify an interaction between H-Ras and IQGAP1 (16, 34). [A recent publication detected IQGAP1 as an interaction partner of M-Ras (47), but M-Ras is not believed to be an important participant in EGF signalling.] We are not aware of studies that have examined a possible association between IQGAP1 and Raf. Another possibility, which may complement the first model, concerns the spatio-temporal regulation of the MAP kinase cascade. It is becoming increasingly apparent that spatio-temporal factors are

important in Ras/MAP kinase signalling (15, 36, 45). Ras and MAP kinases can be targeted to different compartments of the cell. It is conceivable that IQGAP1 may be recruited to a particular subcellular domain by the EGF receptor. Consistent with this hypothesis is the documentation that IQGAP1 can move between different subcellular regions. For example, activation of E-cadherin (25, 29) and antagonism of calmodulin (29) induce IQGAP1 accumulation at the plasma membrane, while increasing intracellular $Ca^{2+}$ concentrations removed IQGAP1 from the cell cortex (32). Further work is necessary to dissect out the intricate molecular mechanisms by which IQGAP1 modulates the MAP kinase cascade.

In the current study, we gain further insight into the participation of IQGAP1 in the MAP kinase signaling cascade. A novel association between IQGAP1 and MEK that modulated MEK activation by EGF was identified. Evidence obtained using diverse experimental approaches strongly supports the concept that IQGAP1 is a scaffold molecule involved in some aspects of MEK-ERK signalling. Finally, a combination of direct *in vitro* binding and co-immunoprecipitation analyses imply that IQGAP1 may enable cells to differentially regulate individual isoforms of MEK/ERK, suggesting that it may contribute to differential signaling in the MAP kinase cascade.

## References


1. **Belanger, L. F., S. Roy, M. Tremblay, B. Brott, A. M. Steff, W. Mourad, P. Hugo, R. Erikson, and J. Charron.** 2003. Mek2 is dispensable for mouse growth and development. Mol Cell Biol **23:**4778-87.

2. **Blagoev, B., I. Kratchmarova, S. E. Ong, M. Nielsen, L. J. Foster, and M. Mann.** 2003. A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. Nat Biotechnol **21:**315-8.

3. **Briggs, M. W., Z. Li, and D. B. Sacks.** 2002. IQGAP1-mediated stimulation of transcriptional co-activation by beta-catenin is modulated by calmodulin. J Biol Chem **277:**7453-65.

4. **Briggs, M. W., and D. B. Sacks.** 2003. IQGAP1 as signal integrator: $Ca^{2+}$, calmodulin, Cdc42 and the cytoskeleton. FEBS Lett **542:**7-11.

5. **Briggs, M. W., and D. B. Sacks.** 2003. IQGAP proteins are integral components of cytoskeletal regulation. EMBO reports **4:**571-574.

6. **Cacace, A. M., N. R. Michaud, M. Therrien, K. Mathes, T. Copeland, G. M. Rubin, and D. K. Morrison.** 1999. Identification of constitutive and ras-inducible phosphorylation sites of KSR: implications for 14-3-3 binding, mitogen-activated protein kinase binding, and KSR overexpression. Mol Cell Biol **19:**229-40.

7. **Castro-Obregon, S., R. V. Rao, G. del Rio, S. F. Chen, K. S. Poksay, S. Rabizadeh, S. Vesce, X. K. Zhang, R. A. Swanson, and D. E. Bredesen.** 2004. Alternative, nonapoptotic programmed cell death: mediation by arrestin 2, ERK2, and Nur77. J Biol Chem **279:**17543-53.

8. **Chang, L., and M. Karin.** 2001. Mammalian MAP kinase signalling cascades. Nature **410**:37-40.

9. **Chen, Z., T. B. Gibson, F. Robinson, L. Silvestro, G. Pearson, B. Xu, A. Wright, C. Vanderbilt, and M. H. Cobb.** 2001. MAP kinases. Chem Rev **101**:2449-76.

10. **Erickson, J. W., R. A. Cerione, and M. J. Hart.** 1997. Identification of an actin cytoskeletal complex that includes IQGAP and the Cdc42 GTPase. J Biol Chem **272**:24443-24447.

11. **Fukata, M., S. Kuroda, K. Fujii, T. Nakamura, I. Shoji, Y. Matsuura, K. Okawa, A. Iwamatsu, A. Kikuchi, and K. Kaibuchi.** 1997. Regulation of cross-linking of actin filament by IQGAP1, a target for Cdc42. J Biol Chem **272**:29579-83.

12. **Fukata, M., T. Watanabe, J. Noritake, M. Nakagawa, M. Yamaga, S. Kuroda, Y. Matsuura, A. Iwamatsu, F. Perez, and K. Kaibuchi.** 2002. Rac1 and Cdc42 capture microtubules through IQGAP1 and CLIP-170. Cell **109**:873-85.

13. **Giroux, S., M. Tremblay, D. Bernard, J. F. Cardin-Girard, S. Aubry, L. Larouche, S. Rousseau, J. Huot, J. Landry, L. Jeannotte, and J. Charron.** 1999. Embryonic death of Mek1-deficient mice reveals a role for this kinase in angiogenesis in the labyrinthine region of the placenta. Curr Biol **9**:369-72.

14. **Gustin, M. C., J. Albertyn, M. Alexander, and K. Davenport.** 1998. MAP kinase pathways in the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev **62**:1264-300.

15. **Hancock, J. F.** 2003. Ras proteins: different signals from different locations. Nat Rev Mol Cell Biol **4:**373-84.

16. **Hart, M. J., M. G. Callow, B. Souza, and P. Polakis.** 1996. IQGAP1, a calmodulin-binding protein with a rasGAP-related domain, is a potential effector for cdc42Hs. EMBO J **15:**2997-3005.

17. **Ho, Y. D., J. L. Joyal, Z. Li, and D. B. Sacks.** 1999. IQGAP1 integrates $Ca^{2+}$/calmodulin and Cdc42 signaling. J Biol Chem **274:**464-70.

18. **Hunter, T.** 2000. Signaling--2000 and beyond. Cell **100:**113-27.

19. **Jaffe, A. B., P. Aspenstrom, and A. Hall.** 2004. Human CNK1 acts as a scaffold protein, linking Rho and Ras signal transduction pathways. Mol Cell Biol **24:**1736-46.

20. **Joyal, J. L., R. S. Annan, Y. D. Ho, M. E. Huddleston, S. A. Carr, M. J. Hart, and D. B. Sacks.** 1997. Calmodulin modulates the interaction between IQGAP1 and Cdc42. Identification of IQGAP1 by nanoelectrospray tandem mass spectrometry. J Biol Chem **272:**15419-25.

21. **Katata, T., K. Irie, A. Fukuhara, T. Kawakatsu, A. Yamada, K. Shimizu, and Y. Takai.** 2003. Involvement of nectin in the localization of IQGAP1 at the cell-cell adhesion sites through the actin cytoskeleton in Madin-Darby canine kidney cells. Oncogene **22:**2097-109.

22. **Kim, S. H., S. K. Lee, and K. Y. Choi.** 1998. Saccharomyces cerevisiae STE11 may contribute to the stabilities of a scaffold protein, STE5, in the pheromone signaling pathway. Mol Cells **8:**130-7.

23. **Kolch, W.** 2000. Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem J **351 Pt 2:**289-305.

24. **Kortenjann, M., and P. E. Shaw.** 1995. Raf-1 kinase and ERK2 uncoupled from mitogenic signals in rat fibroblasts. Oncogene **11:**2105-12.

25. **Kuroda, S., M. Fukata, M. Nakagawa, K. Fujii, T. Nakamura, T. Ookubo, I. Izawa, T. Nagase, N. Nomura, H. Tani, I. Shoji, Y. Matsuura, S. Yonehara, and K. Kaibuchi.** 1998. Role of IQGAP1, a target of the small GTPases Cdc42 and Rac1, in regulation of E-cadherin- mediated cell-cell adhesion. Science **281:**832-5.

26. **Levchenko, A., J. Bruck, and P. W. Sternberg.** 2000. Scaffold proteins may biphasically affect the levels of mitogen-activated protein kinase signaling and reduce its threshold properties. Proc Natl Acad Sci U S A **97:**5818-23.

27. **Lewis, T. S., P. S. Shapiro, and N. G. Ahn.** 1998. Signal transduction through MAP kinase cascades. Adv Cancer Res **74:**49-139.

28. **Li, W., M. Han, and K. L. Guan.** 2000. The leucine-rich repeat protein SUR-8 enhances MAP kinase activation and forms a complex with Ras and Raf. Genes Dev **14:**895-900.

29. **Li, Z., S. H. Kim, J. M. Higgins, M. B. Brenner, and D. B. Sacks.** 1999. IQGAP1 and calmodulin modulate E-cadherin function. J Biol Chem **274:**37885-92.

30. **Mansour, S. J., W. T. Matten, A. S. Hermann, J. M. Candia, S. Rong, K. Fukasawa, G. F. Vande Woude, and N. G. Ahn.** 1994. Transformation of

mammalian cells by constitutively active MAP kinase kinase. Science **265:**966-70.

31. **Mataraza, J. M., M. W. Briggs, Z. Li, A. Entwistle, A. J. Ridley, and D. B. Sacks.** 2003. IQGAP1 promotes cell motility and invasion. J Biol Chem **278:**41237-45.

32. **Mateer, S. C., A. E. McDaniel, V. Nicolas, G. M. Habermacher, M. J. Lin, D. A. Cromer, M. E. King, and G. S. Bloom.** 2002. The mechanism for regulation of the F-actin binding activity of IQGAP1 by calcium/calmodulin. J Biol Chem **277:**12324-33.

33. **Mbele, G. O., J. C. Deloulme, B. J. Gentil, C. Delphin, M. Ferro, J. Garin, M. Takahashi, and J. Baudier.** 2002. The zinc- and calcium-binding S100B interacts and co-localizes with IQGAP1 during dynamic rearrangement of cell membranes. J Biol Chem **277:**49998-50007.

34. **McCallum, S. J., W. J. Wu, and R. A. Cerione.** 1996. Identification of a putative effector for Cdc42Hs with high sequence similarity to the RasGAP-related protein IQGAP1 and a Cdc42Hs binding partner with similarity to IQGAP2. J Biol Chem **271:**21732-37.

35. **Meighan-Mantha, R. L., A. Wellstein, and A. T. Riegel.** 1997. Differential regulation of extracellular signal-regulated kinase 1 and 2 activity during 12-O-tetradecanoylphorbol 13-acetate-induced differentiation of HL-60 cells. Exp Cell Res **234:**321-8.

36. **Morrison, D. K., and R. J. Davis.** 2003. Regulation of MAP kinase signaling modules by scaffold proteins in mammals. Annu Rev Cell Dev Biol **19:**91-118.

37. **Nguyen, A., W. R. Burack, J. L. Stock, R. Kortum, O. V. Chaika, M. Afkarian, W. J. Muller, K. M. Murphy, D. K. Morrison, R. E. Lewis, J. McNeish, and A. S. Shaw.** 2002. Kinase suppressor of Ras (KSR) is a scaffold which facilitates mitogen-activated protein kinase activation in vivo. Mol Cell Biol **22:**3035-45.

38. **Park, S. H., A. Zarrinpar, and W. A. Lim.** 2003. Rewiring MAP kinase pathways using alternative scaffold assembly mechanisms. Science **299:**1061-4.

39. **Robbins, D. J., E. Zhen, H. Owaki, C. A. Vanderbilt, D. Ebert, T. D. Geppert, and M. H. Cobb.** 1993. Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. J Biol Chem **268:**5097-106.

40. **Roy, M., Z. Li, and D. B. Sacks.** 2004. IQGAP1 binds ERK2 and modulates its activity. J Biol Chem **279:**17329-37.

41. **Ruiz-Velasco, R., C. C. Lanning, and C. L. Williams.** 2002. The activation of Rac1 by M3 muscarinic acetylcholine receptors involves the translocation of Rac1 and IQGAP1 to cell junctions and changes in the composition of protein complexes containing Rac1, IQGAP1, and actin. J Biol Chem **277:**33081-91.

42. **Schaeffer, H. J., A. D. Catling, S. T. Eblen, L. S. Collier, A. Krauss, and M. J. Weber.** 1998. MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science **281:**1668-71.

43. **Sokol, S. Y., Z. Li, and D. B. Sacks.** 2001. The effect of IQGAP1 on *Xenopus* embryonic ectoderm requires Cdc42. J Biol Chem **276:**48425-30.

44. **Swart-Mataraza, J. M., Z. Li, and D. B. Sacks.** 2002. IQGAP1 is a component of Cdc42 signaling to the cytoskeleton. J Biol Chem **277:**24753-63.

45. **Torii, S., M. Kusakabe, T. Yamamoto, M. Maekawa, and E. Nishida.** 2004. Sef is a spatial regulator for Ras/MAP kinase signaling. Dev Cell **7**:33-44.

46. **Ussar, S., and T. Voss.** 2004. MEK1 and MEK2, Different Regulators of the G1/S Transition. J Biol Chem **279**:43861-43869.

47. **Vasilescu, J., X. Guo, and J. Kast.** 2004. Identification of protein-protein interactions using in vivo cross-linking and mass spectrometry. Proteomics **4**:3845-54.

48. **Vomastek, T., H. J. Schaeffer, A. Tarcsafalvi, M. E. Smolkin, E. A. Bissonette, and M. J. Weber.** 2004. Modular construction of a signaling scaffold: MORG1 interacts with components of the ERK cascade and links ERK signaling to specific agonists. Proc Natl Acad Sci U S A **101**:6981-6.

49. **Watanabe, T., S. Wang, J. Noritake, K. Sato, M. Fukata, M. Takefuji, M. Nakagawa, N. Izumi, T. Akiyama, and K. Kaibuchi.** 2004. Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. Dev Cell **7**:871-83.

50. **Xu, B., J. L. Wilsbacher, T. Collisson, and M. H. Cobb.** 1999. The N-terminal ERK-binding site of MEK1 is required for efficient feedback phosphorylation by ERK2 in vitro and ERK activation in vivo. J Biol Chem **274**:34029-35.

51. **Yeung, K., T. Seitz, S. Li, P. Janosch, B. McFerran, C. Kaiser, F. Fee, K. D. Katsanakis, D. W. Rose, H. Mischak, J. M. Sedivy, and W. Kolch.** 1999. Suppression of Raf-1 kinase activity and MAP kinase signalling by RKIP. Nature **401**:173-7.

## Footnotes

‡ This study was supported in part by grants from the National Institutes of Health (to D.B.S.) and an United States Army Fellowship grant DAMD17-02-1-0304 (to M.R.).

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The abbreviations used are:

MEK, MAPK/ERK kinase; ERK, extracellular-signal-regulated kinase; KSR, kinase suppressor of Ras; MAP kinase, mitogen-activated protein kinase; MORG1, MAP kinase organizer 1; EGF, epidermal growth factor; GST, glutathione *S*-transferase; CHD, calponin homology domain; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; $Ni^{2+}$-NTA, nickel-nitriotriacetic acid.

**Acknowledgements**

We are grateful to Natalie Ahn (University of Colorado) for generously donating the HA-tagged and $His_6$-tagged MEK1 and MEK2 constructs, to Melanie Cobb (University of Texas) for the gift of $His_6$-tagged ERK1 and ERK2 and to David Turner (University of Michigan) for the mU6pro vector. We thank Chris French for the use of Nucleofector (Amaxa) and Angela Lam for technical assistance. We appreciate Rob Krikorian's expert help in the preparation of the manuscript.

## Figure Legends

FIG. 1. **MEK1 and MEK2 bind to IQGAP1.** *A*, GST alone or GST-IQGAP1 (IQGAP1) bound to glutathione-Sepharose was incubated with purified $His_6$-MEK1 (panel A) or $His_6$-MEK2 (panel B). Complexes were isolated and washed as described under "Experimental Procedures". 100 ng of purified His-tagged protein (MEK1 in panel A or MEK2 in panel B) was loaded as input. After samples were resolved by SDS-PAGE, the gel was cut into two pieces; the top portion (containing IQGAP1) was stained with Coomassie Blue, whereas the bottom half was transferred to PVDF membrane and probed with anti-MEK antibody. The positions of migration of MEK1 and MEK2 are depicted. The data are representative of six independent experiments.

FIG. 2. **MEK1, MEK2 and ERK1 co-immunoprecipitate with IQGAP1.** *A,* MCF-7 cells were transiently transfected with pMCL-MKK1 (MEK1) or pMCL-MKK2 (MEK2). Twenty-four hours later, cells were starved of serum overnight, then incubated with vehicle (-) or 100 ng/ml EGF (+) for 10 min. After lysis, equal amounts of protein were incubated with non-immune rabbit serum (NIRS) or immunoprecipitated (IP) with anti-IQGAP1 polyclonal antibody. Both immune complexes (IP*)* and unfractionated lysates (Lysate) were resolved by SDS-PAGE. After transfer to PVDF membranes, the blots were probed with monoclonal anti-IQGAP1 and anti-MEK antibodies. Data are representative of two independent experiments for MEK1 and four independent experiments for MEK2. *B*, untransfected MCF-7 cells were starved of serum for sixteen hours, then incubated with vehicle (-) or 100 ng/ml EGF (+) for 10 min. Cells were

exposed to a crosslinker, DSP, before lysis. Equal amounts of protein lysate were immunoprecipitated (IP) as described for panel A. Western blots were probed with anti-IQGAP1 and anti-ERK1 antibodies. Data are representative of three independent experiments.

FIG. 3. **IQGAP1 modulates EGF-stimulated activation of MEK.** *A,* MCF/V, MCF/I and MCF-siIQ8 (siIQ8) cells were starved of serum overnight and treated with vehicle or 100 ng/ml EGF for 10 min. Equal amounts of protein from the cell lysates were resolved by SDS-PAGE and Western blots were probed with anti-IQGAP1 and anti-phospho-MEK antibodies. The membranes were stripped and reprobed with anti-MEK antibody. *B,* the amount of phospho-MEK was quantified by densitometry and corrected for the amount of MEK in the corresponding lysate. Data, expressed relative to the amount of phospho-MEK in vehicle-treated MCF/V cells, represent the means ± S.E. ($n$=3). *, significantly different from vehicle-treated MCF/V cells ($p<0.001$).

FIG. 4. **Altering intracellular IQGAP1 concentrations reduces EGF-stimulated MEK activity.** *A,* MCF-7 cells were transiently transfected with vectors mU6pro (mU6) or pcDNA3 (pcDNA), IQGAP1 (WT) or siRNAs for IQGAP1 (siIQ5 and siIQ8). Cells were processed as described for Fig. 3A. *B,* the amount of phospho-MEK was quantified by densitometry and corrected for the amount of MEK in the corresponding lysate. Data, expressed relative to the amount of phospho-MEK in cells transfected with the appropriate vector, represent the means ± S.E. ($n$=3). *, significantly different from mU6-transfected cells ($p<0.001$); †, significantly different from pcDNA-transfected cells ($p<0.01$).

FIG. 5. **Schematic representation of IQGAP1 constructs.** FIG. 5. **Identification of amino acid residues of IQGAP1 necessary for binding MEK.** Schematic representation of IQGAP1 constructs depicting full length IQGAP1, truncated IQGAP1 fragments and deletion mutants. The identified protein interaction motifs and the specific amino acid residues absent from each mutant are indicated. *CHD,* calponin homology domain; *WW*, poly-proline binding domain; *IQ*, four tandem calmodulin-binding motifs; IQGAP1-N, N-terminal half of IQGAP1; IQGAP1-C, C-terminal half of IQGAP1; IQGAP1-N1, comprising amino acids 2-431 and IQGAP1-N2, harbouring amino acids 432-863.

FIG. 6. **Identification of amino acid residues of IQGAP1 necessary for binding MEK.** *A* and *B,* [$^{35}$S]methionine-labelled pcDNA vector (V), wild type IQGAP1 (WT), IQGAP1-C (C), IQGAP1-N (N), IQGAP1-N1 (N1), IQGAP2-N2 (N2), IQGAP1ΔCHD (ΔCHD), IQGAP1ΔIQ (ΔIQ) and IQGAP1ΔWW (ΔWW), produced with the TNT Quick Coupled Transcription/Translation system were incubated with $Ni^{2+}$-NTA beads (last lane in panels A and B) or equal amounts of $His_6$-MEK1 (panel A) or $His_6$-MEK2 (panel B). Complexes were isolated with $Ni^{2+}$-NTA agarose beads and resolved by SDS-PAGE. Gels were dried and processed by autoradiography. Data are representative of three independent experiments. *C*, an aliquot of [$^{35}$S]methionine-labelled TNT product (equivalent to 10% of the amount that was subjected to pulldown) for the $His_6$-MEK pulldown assays was resolved by SDS-PAGE, dried and processed by autoradiography (Input). Data are representative of three independent experiments.

FIG. 7. **Binding to MEK is necessary for IQGAP1 to alter EGF-stimulated MEK-ERK signalling.** *A,* HEK-293H cells were transiently transfected with pcDNA3 vector (V), IQGAP1 (WT), IQGAP1ΔIQ (ΔIQ) or IQGAP1ΔWW (ΔWW). Cells were starved of serum for 16 h, followed by incubation with 100 ng/ml EGF for 10 min. Equal amounts of protein from the cell lysates were resolved by SDS-PAGE, transferred to PVDF membrane and probed with anti-myc antibody (Myc-IQGAP1) (the transfected IQGAP1 constructs are myc-tagged) and an antibody specific for phosphorylated MEK isoforms (phospho-MEK). The membrane was stripped and reprobed with anti-IQGAP1 (recognizes both endogenous and transfected IQGAP1) and anti-MEK antibodies. Data are representative of four independent experiments. *B,* the amount of phosphorylated MEK isoforms (phospho-MEK) was quantified by densitometry and corrected for the amount of total MEK in the corresponding lysate. Data, expressed relative to the amount of phospho-MEK in cells transfected with pcDNA3 vector, represent the means ± S.E. ($n$=4). *, significantly different from vector-transfected cells ($p<0.01$); **, significantly different from vector-transfected cells ($p<0.001$).

FIG. 8. **Deletion of residues 699-905 of IQGAP1 has no effect on IQGAP1-mediated attenuation of EGF-stimulated ERK activity.** *A,* HEK-293H cells were transiently transfected and processed as described in Fig. 7A. Western blots were probed with anti-myc antibody (Myc-IQGAP1) and an antibody specific for phosphorylated ERK isoforms (phospho-ERK). The membrane was stripped and reprobed with anti-IQGAP1 and anti-ERK antibodies. Data are representative of three independent experiments. *B,* the amount of phosphorylated ERK isoforms (phospho-ERK) was quantified by densitometry and corrected for the amount of total ERK in the corresponding lysate. Data, expressed

relative to the amount of phospho-ERK in cells transfected with pcDNA3 vector, represent the means ± S.E. ($n$=4 for V, WT and ΔWW and $n$=3 for ΔIQ). *, significantly different from vector-transfected cells ($p<0.05$).

FIG. 9. **ERK1 facilitates MEK binding to IQGAP1.** *A*, GST-IQGAP1 or GST alone bound to glutathione-Sepharose was incubated with purified $His_6$-MEK1, $His_6$-ERK1 or both MEK1 and ERK1. Complexes were isolated and washed as described under "Experimental Procedures". After samples were resolved by SDS-PAGE, the gel was cut into two pieces; the top portion (containing IQGAP1) was stained with Coomassie Blue, whereas the bottom half was transferred to PVDF membrane and probed with anti-MEK and anti-ERK antibodies. The positions of migration of MEK1 and ERK1 are depicted. The data are representative of three independent experiments. The amounts of MEK1 and ERK1 bound to IQGAP1 were quantified by densitometry. Data, expressed relative to the amount of MEK1 in samples without ERK1 (■) and ERK1 without MEK1 (□), represent the means ± S.E. ($n$=6). *, significantly different from MEK1 alone ($p<0.05$). *B, in vitro* binding was performed as described in panel A using MEK2 and ERK1. *, significantly different from MEK2 alone ($p<0.001$).

FIG. 10. **ERK2 facilitates MEK2 binding to IQGAP1.** *In vitro* binding was performed as described in Fig. 9 using MEK2 and ERK2 (panel A) and MEK1 and ERK2 (panel B); Data, expressed relative to the amount of MEK1 in samples without ERK1 (■) and ERK1 without MEK1 (□), represent the means ± S.E. ($n$=3). *, significantly different from MEK2 alone ($p<0.0001$).

FIG. 11. **IQGAP1 modulates MEK and ERK activation in a concentration-dependent manner.** Data from EGF-treated MCF-7 cells were analysed to compare the extent of phosphorylation of MEK and ERK in cells expressing different concentrations of IQGAP1. The Y-axes represent relative phosphorylation of MEK (●) (phospho-MEK) and ERK (□) (phospho-ERK), corrected for total MEK and ERK, respectively. The X-axis represents the relative amounts of IQGAP1. Vector-transfected cells are set at 1.0.

FIG. 12. **Model of IQGAP1 functioning as a scaffold in the MAP kinase pathway.** *A,* When the concentration of the scaffolding protein, IQGAP1, is too low, functional IQGAP1 complexes containing MEK and ERK are unable to form. *B,* at the optimal scaffold concentration, the kinases are in an appropriate stoichiometric ratio with IQGAP1. Functional MAP kinase signaling complexes are formed. *C,* when the concentration of IQGAP1 is excessive, many different IQGAP1 molecules harbouring only one of the components of the cascade form complexes, which cannot function optimally. The binding site for ERK2 on IQGAP1 is the region containing the poly-proline-binding domain (WW) and that of MEK is the region spanning the calmodulin-binding motif (IQ). Raf binding to IQGAP1 has not been documented.

Fig. 1

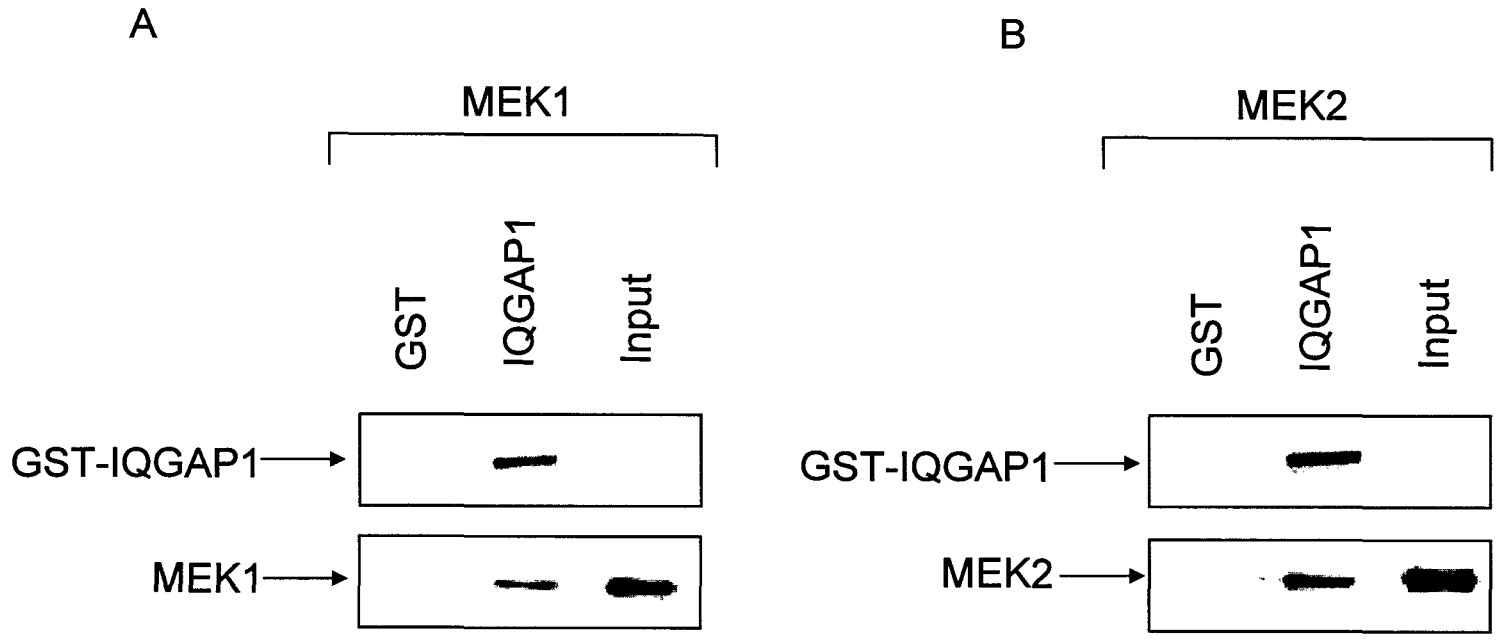

A
MEK1
GST
IQGAP1
Input
GST-IQGAP1
MEK1
B
MEK2
GST
IQGAP1
Input
GST-IQGAP1
MEK2

Fig. 2A

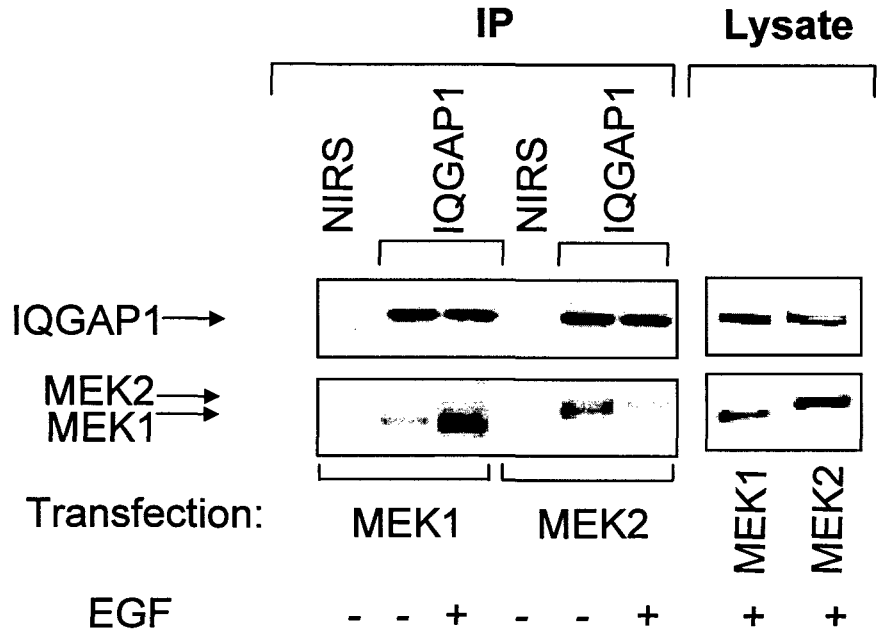

IP
Lysate
NIRS
IQGAP1
NIRS
IQGAP1
IQGAP1
MEK2
MEK1
Transfection:
MEK1
MEK2
MEK1
MEK2
EGF
- - + - - +
+
+

Fig. 2B

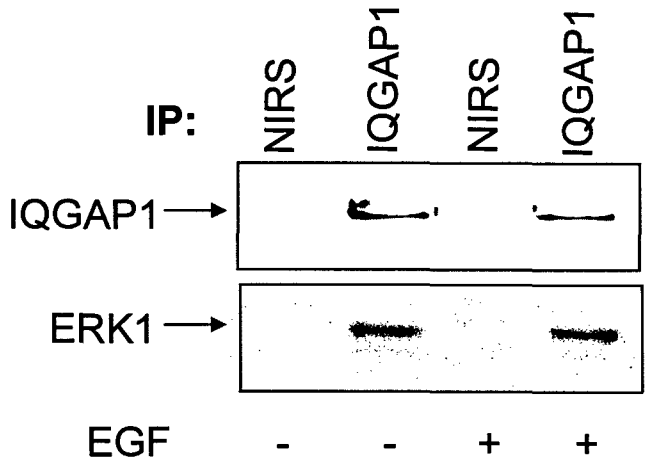


Fig. 3A

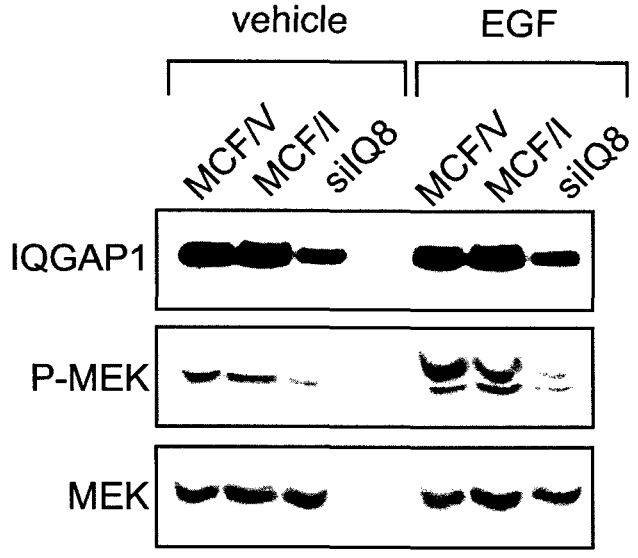


Fig. 3 B

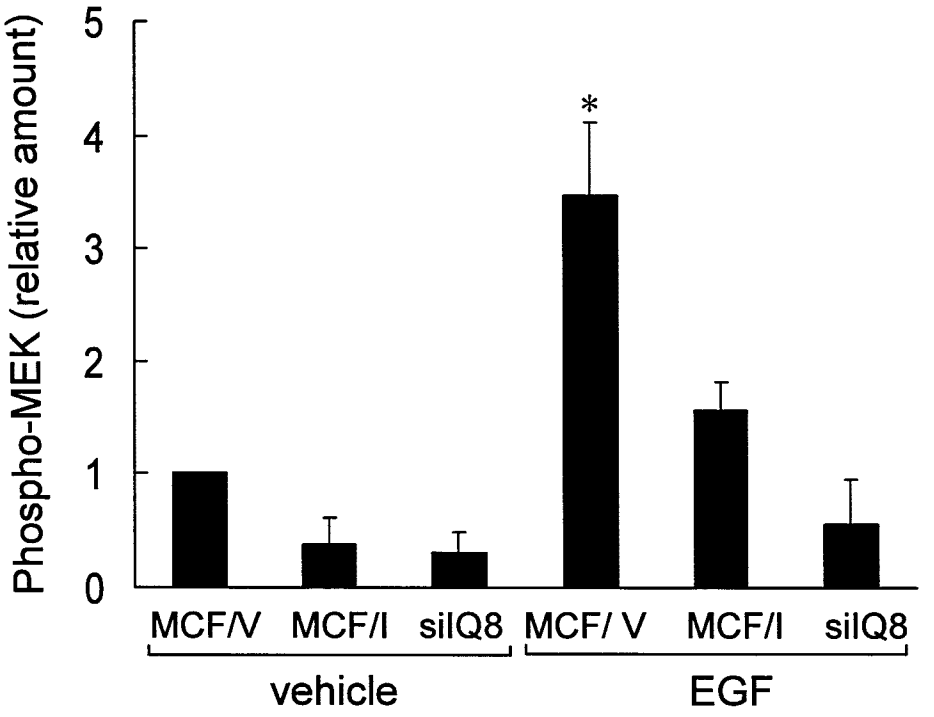
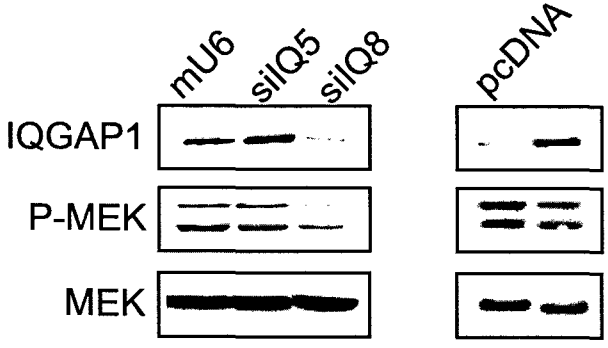

Phospho-MEK (relative amount)
5
4
3
2
1
0
*
MCF/V
MCF/I
siIQ8
MCF/ V
MCF/I
siIQ8
vehicle
EGF

Fig. 4

A


mU6
siIQ5
siIQ8
pcDNA
IQGAP1
P-MEK
MEK

B

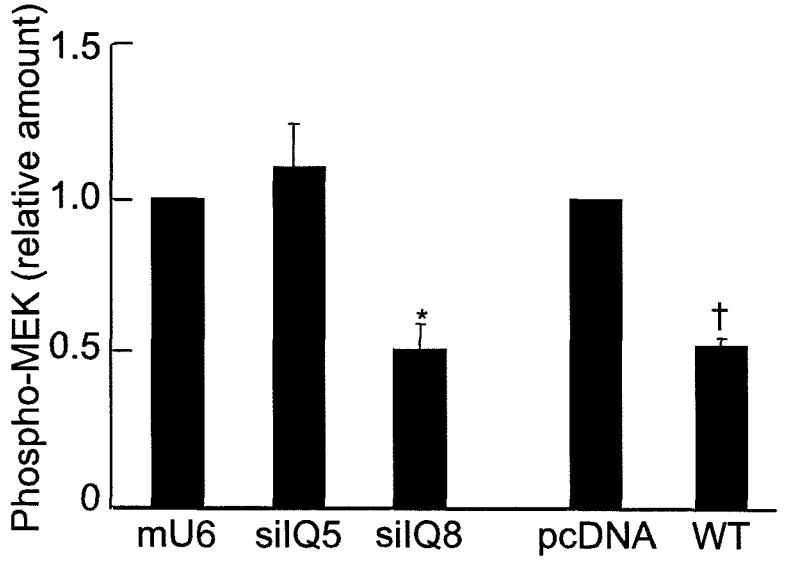

Phospho-MEK (relative amount)
1.5
1.0
0.5
0
*
†
mU6
siIQ5
siIQ8
pcDNA
WT

Fig. 5

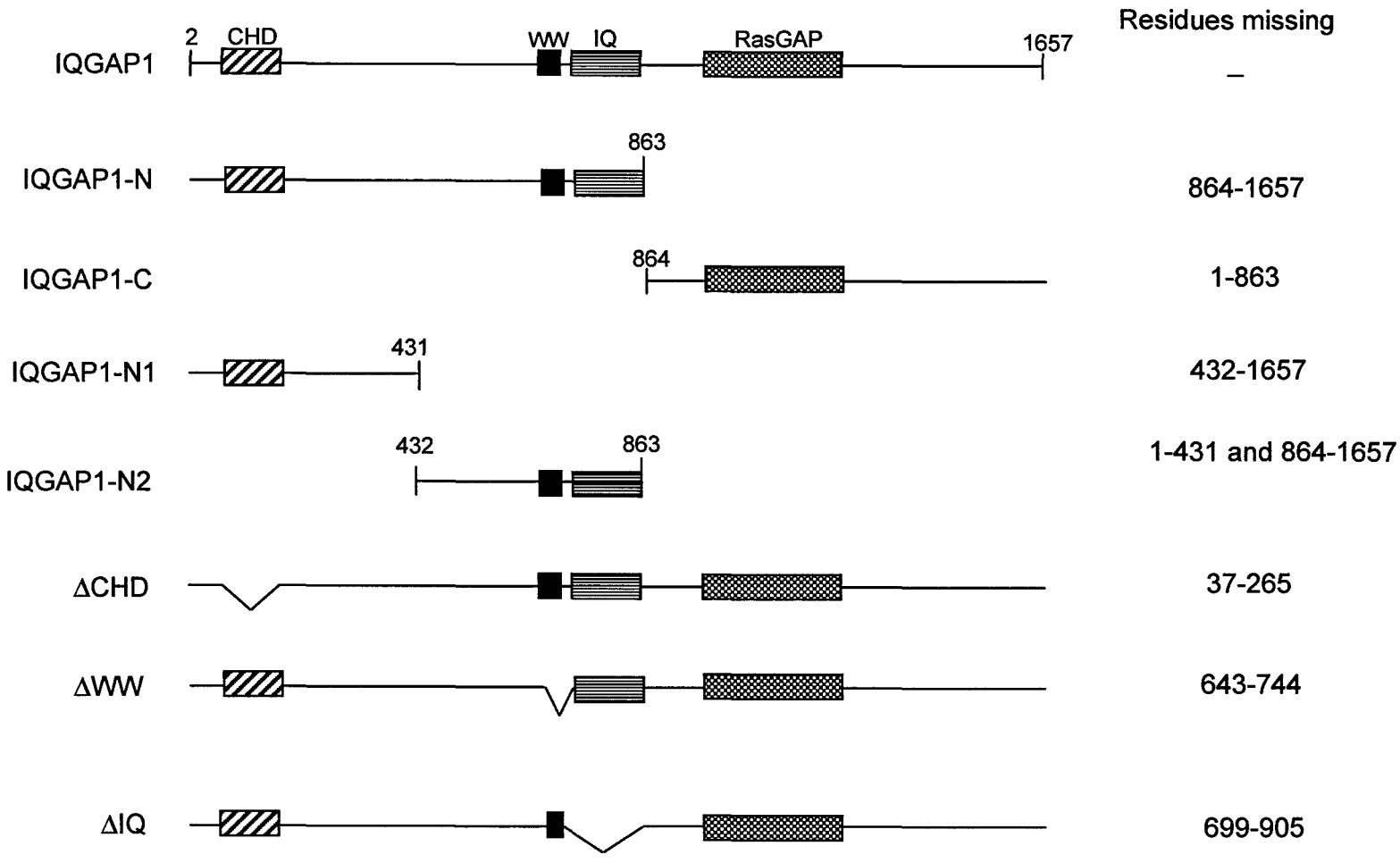
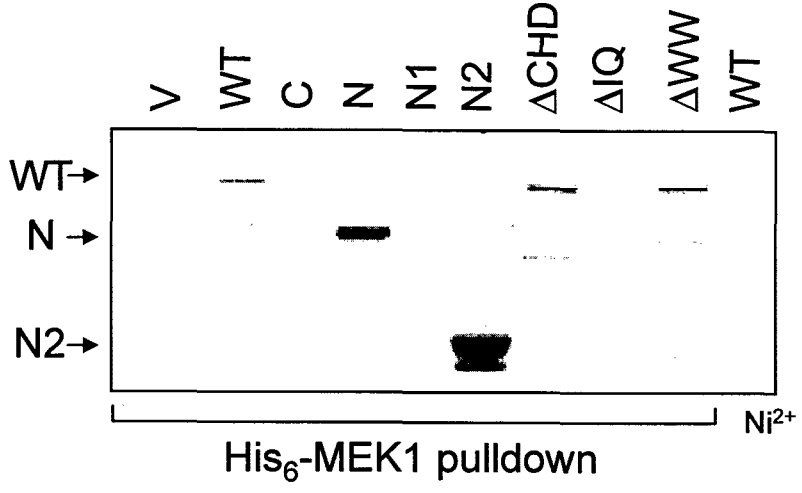
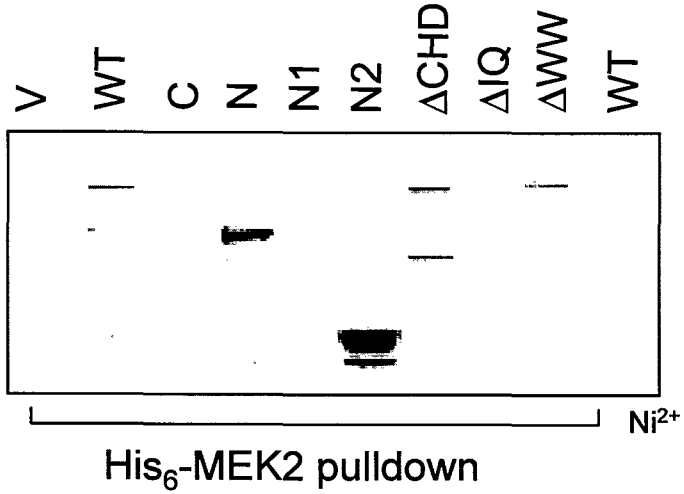

Residues missing
IQGAP1
2
CHD
WW
IQ
RasGAP
1657
–
IQGAP1-N
863
864-1657
IQGAP1-C
864
1-863
IQGAP1-N1
431
432-1657
IQGAP1-N2
432
863
1-431 and 864-1657
ΔCHD
37-265
ΔWW
643-744
ΔIQ
699-905

Fig. 6

A


V
WT
C
N
N1
N2
ΔCHD
ΔIQ
ΔWW
WT
WT
N
N2
Ni2+
His6-MEK1 pulldown

B


V
WT
C
N
N1
N2
ΔCHD
ΔIQ
ΔWW
WT
Ni2+
His6-MEK2 pulldown

C

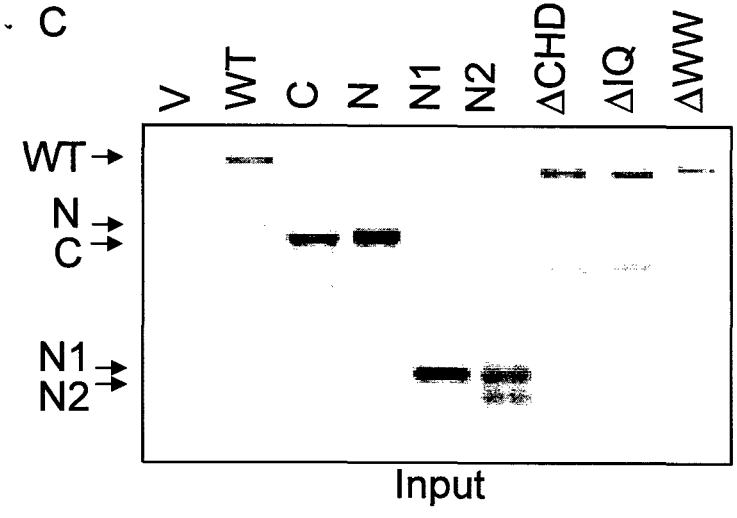

V
WT
C
N
N1
N2
ΔCHD
ΔIQ
ΔWW
WT
N
C
N1
N2
Input

Fig. 7A

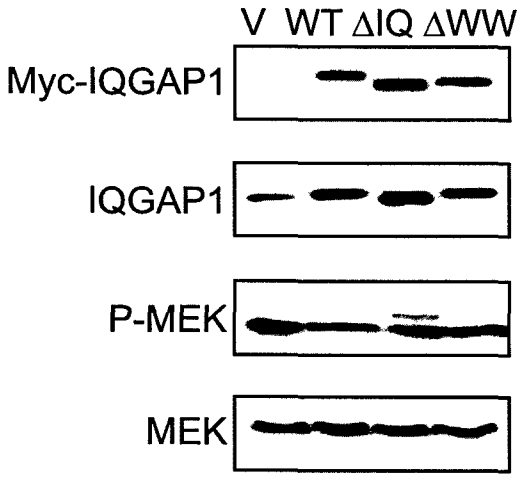


Fig. 7B

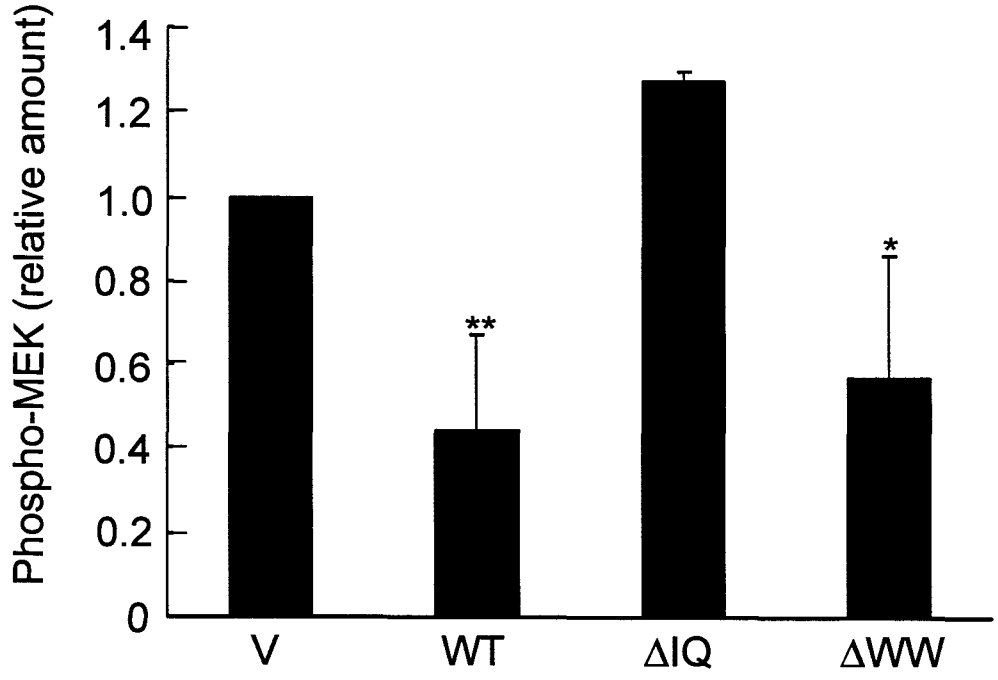


Fig. 8A

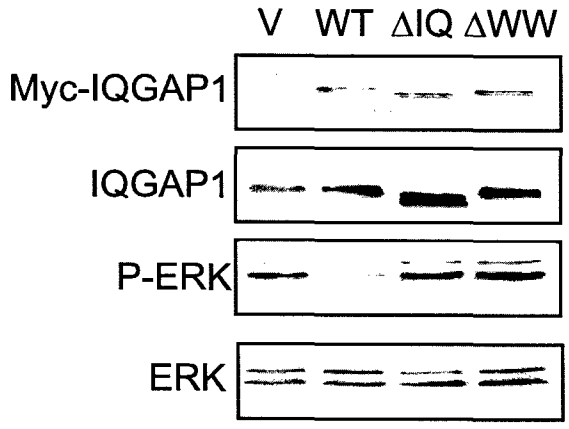


Fig. 8B

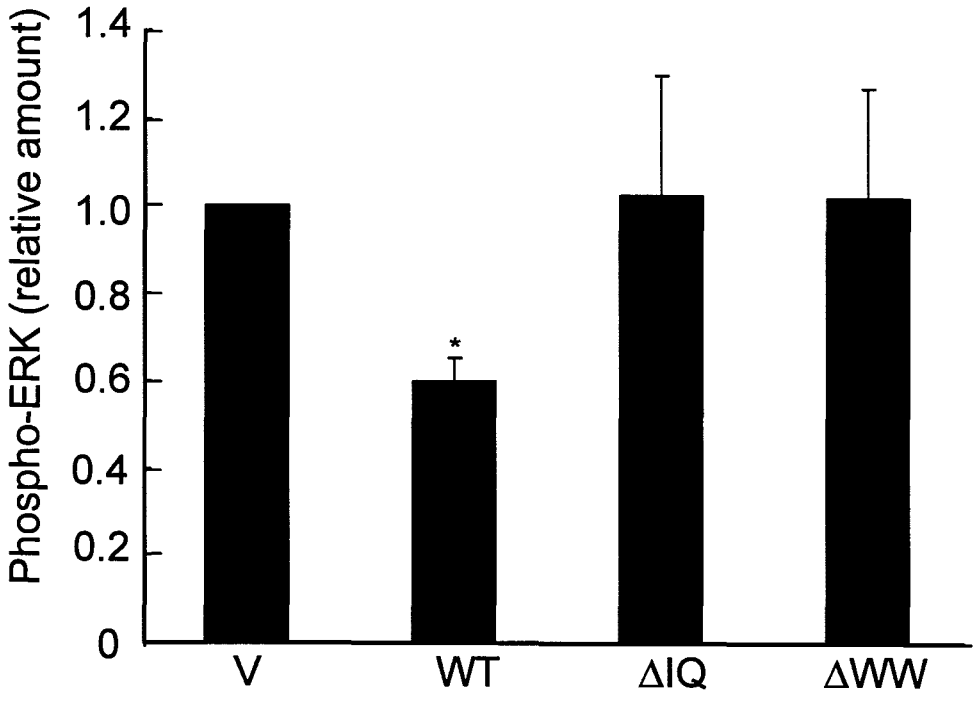


Fig. 9 A

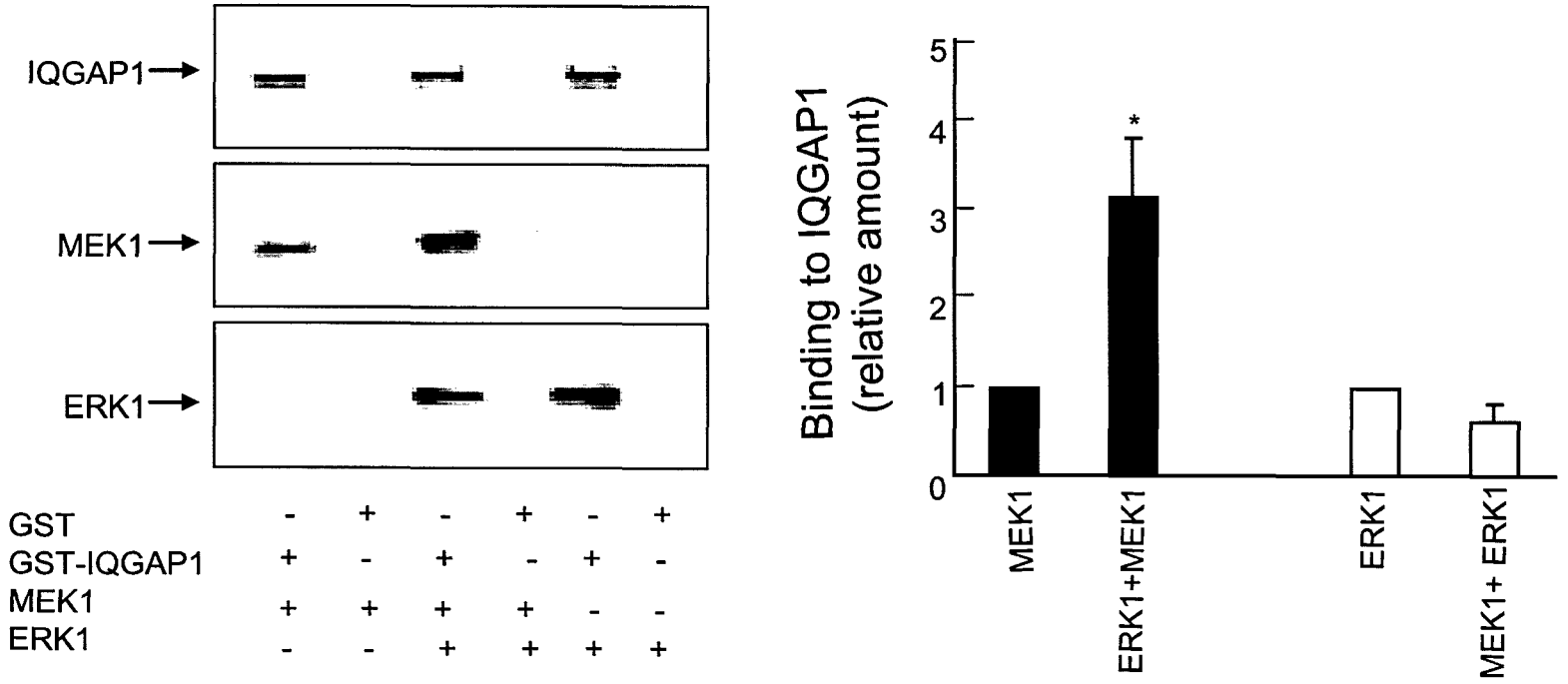
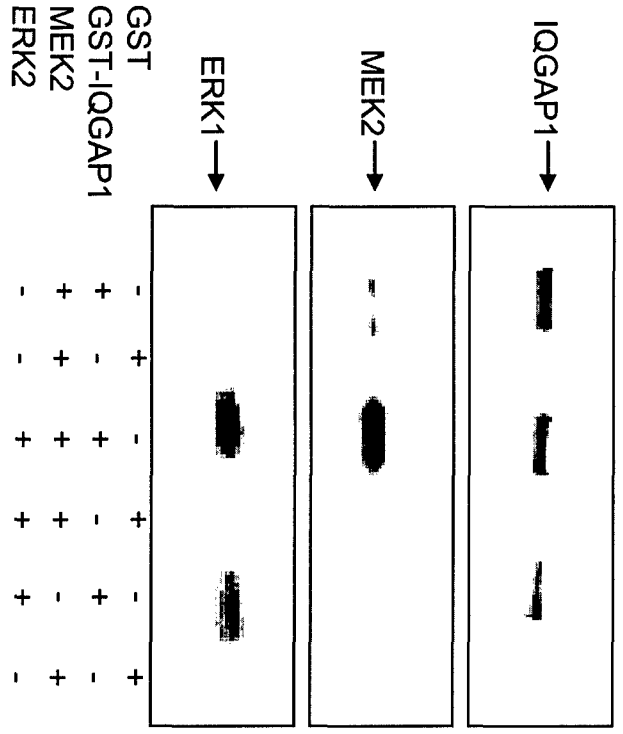

IQGAP1
MEK1
ERK1
GST - + - + - +
GST-IQGAP1 + - + - + -
MEK1 + + + + - -
ERK1 - - + + + +
Binding to IQGAP1 (relative amount)
0
1
2
3
4
5
*
MEK1
ERK1+MEK1
ERK1
MEK1+ ERK1

Fig. 9 B


GST
GST-IQGAP1
MEK2
ERK2
ERK1
MEK2
IQGAP1

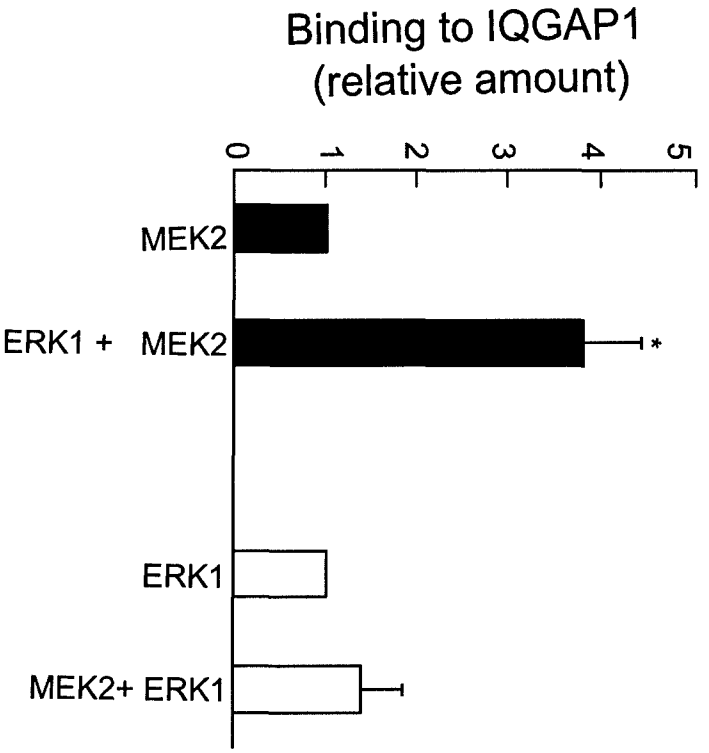

Binding to IQGAP1
(relative amount)
0
1
2
3
4
5
MEK2
ERK1 + MEK2
*
ERK1
MEK2+ ERK1

Fig. 10 A

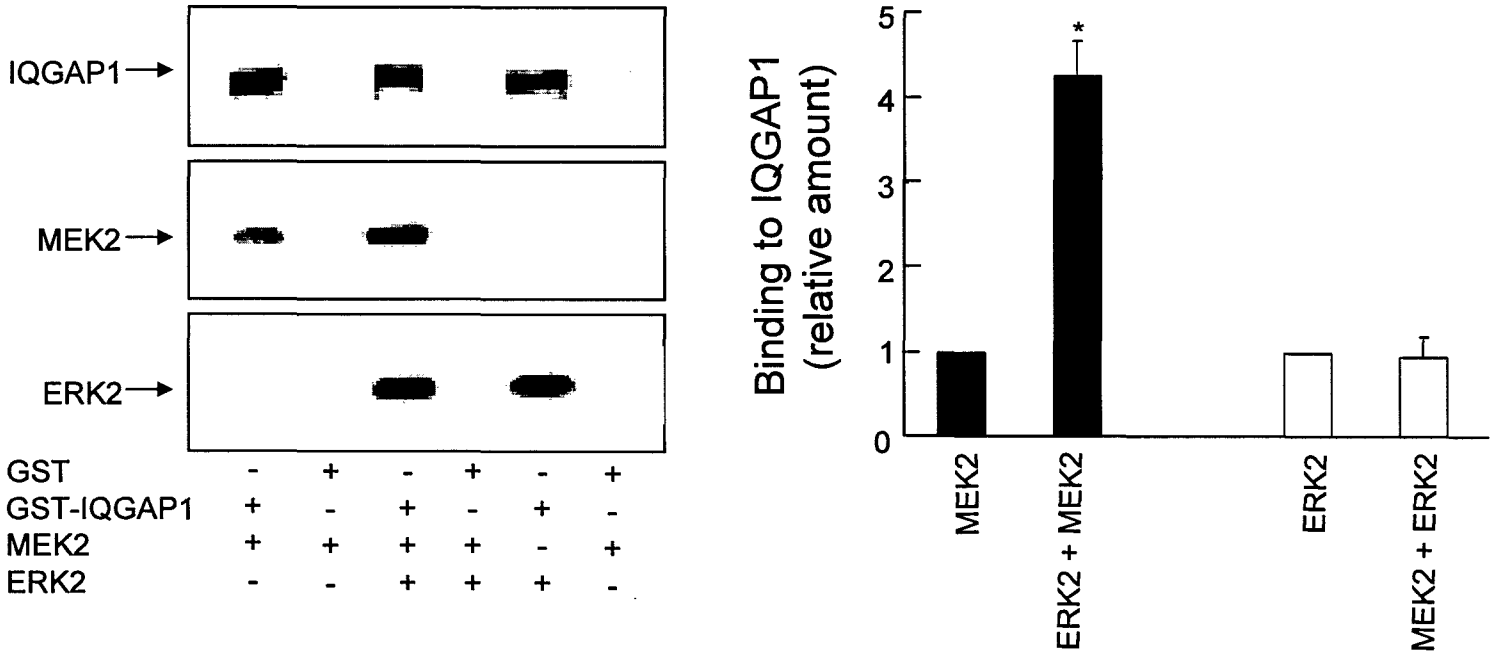
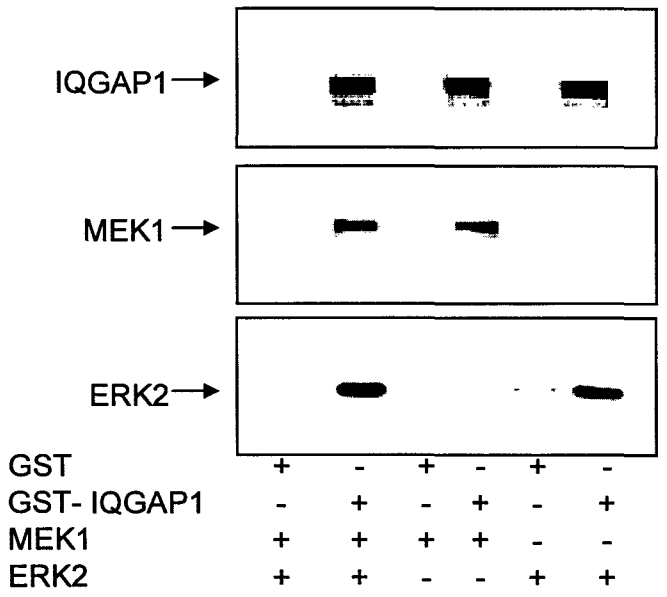

IQGAP1
MEK2
ERK2
GST - + - + - +
GST-IQGAP1 + - + - + -
MEK2 + + + + - +
ERK2 - - + + + -
Binding to IQGAP1 (relative amount)
0
1
2
3
4
5
*
MEK2
ERK2 + MEK2
ERK2
MEK2 + ERK2

Fig. 10 B


IQGAP1
MEK1
ERK2
GST + - + - + -
GST- IQGAP1 - + - + - +
MEK1 + + + + - -
ERK2 + + - - + +

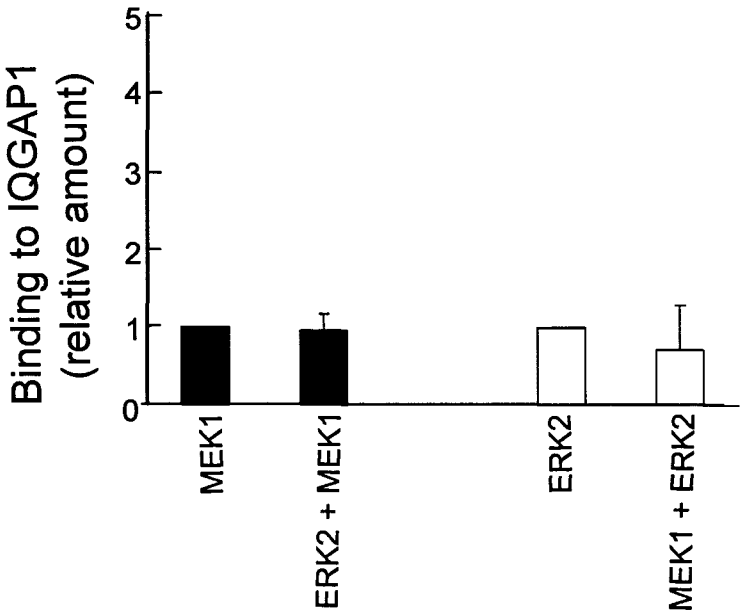

Binding to IQGAP1
(relative amount)
0
1
2
3
4
5
MEK1
ERK2 + MEK1
ERK2
MEK1 + ERK2

Fig. 11

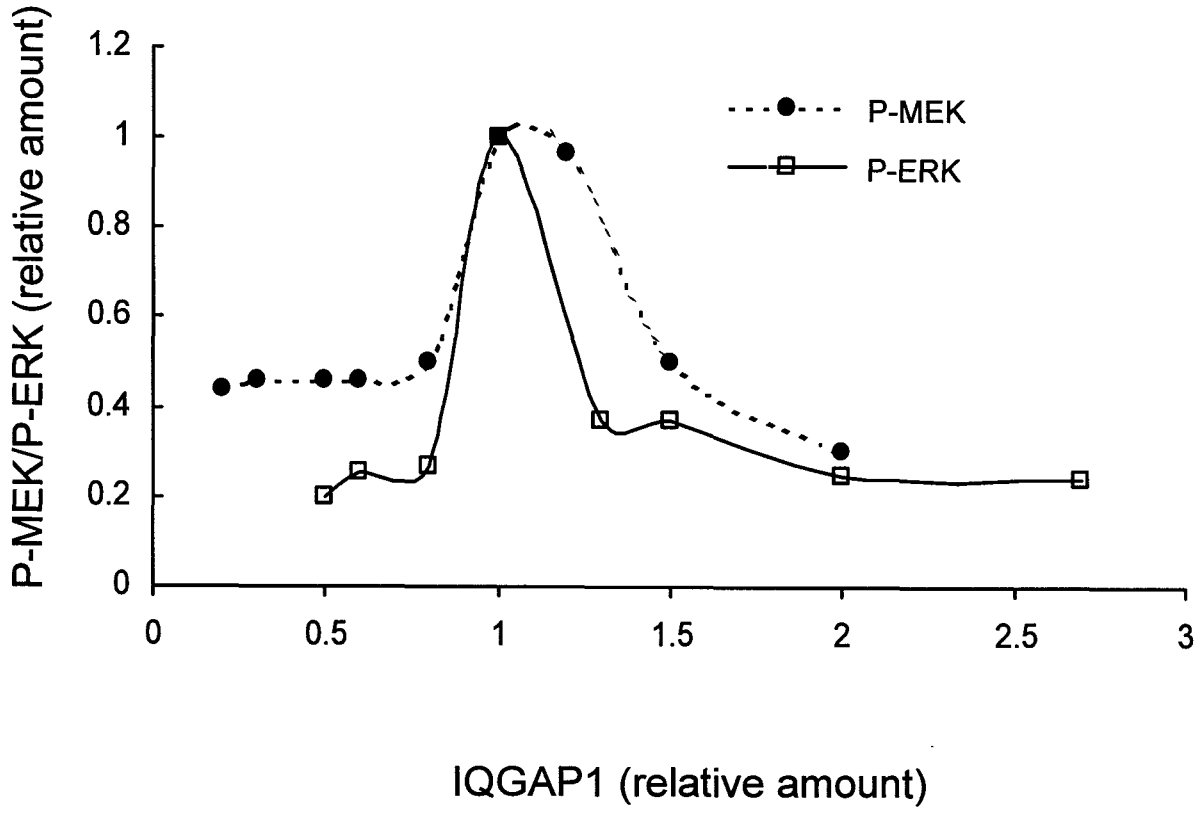
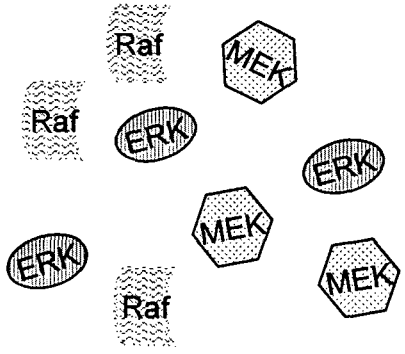
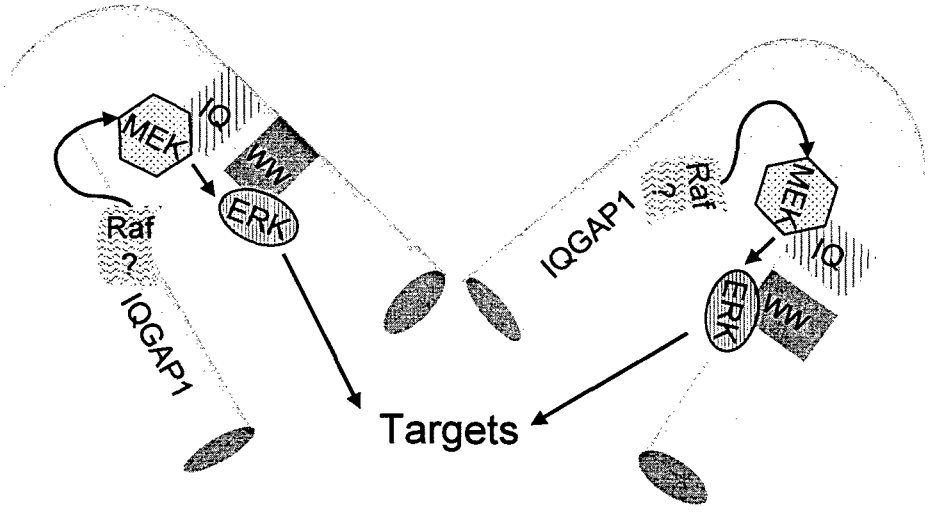

P-MEK/P-ERK (relative amount)
1.2
1
0.8
0.6
0.4
0.2
0
0
0.5
1
1.5
2
2.5
3
P-MEK
P-ERK
IQGAP1 (relative amount)

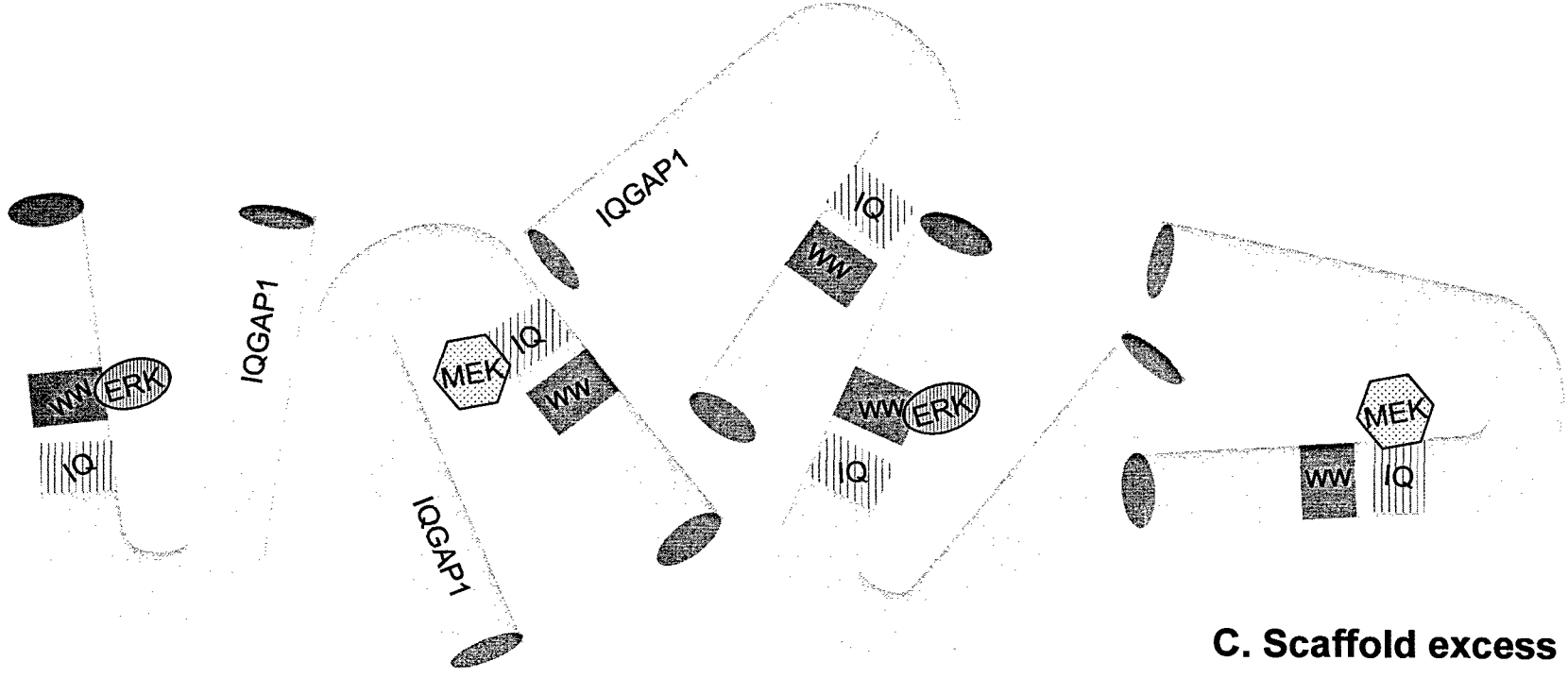
Fig. 12




**A. Scaffold deficiency**




**B. Optimal scaffold:kinase**




**C. Scaffold excess**